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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

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(54) Title: COMPOSITIONS AND METHODS FOR TARGETING PEPTIDES IN HUMANS *IN VIVO*(57) Abstract: The present invention concerns methods and compositions for identifying human targeting peptides sequences. In preferred embodiments, a primary phage library is administered to a human *in vivo* and phage bearing targeting peptides are collected from selected organs, tissues or cells types. In more preferred embodiments, collected phage are amplified, pooled and administered to a second human for another round of selection. The peptides are of use for targeted delivery of therapeutic agents, including gene therapy vectors. Such targeted delivery may be used for detection, diagnosis or treatment of human diseases. In certain embodiments, the peptide may be attached to an imaging agent and administered to a human to obtain an image or to diagnose a disease state. Also disclosed are a large number of targeting peptide sequences and consensus motifs that are selective for human organs or tissues, obtained by the methods of the present invention.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER IPC(7) : Please See Extra Sheet. US CL : 455/5, 69.1, 71.1; 424/9.1, 9.541; 550/300, 550 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 455/5, 69.1, 71.1; 424/9.1, 9.541; 550/300, 550 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN: CAPLUS, MDLINE, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PASQUALINI, R. et al. Organ Targeting in Vivo Using Phage Display Peptide Libraries. Nature. March 1996, Vol. 380, pages 364-366, see entire document.	1-14 and 61-63
Y	ROJOTTE, D. et al. Molecular Heterogeneity of the Vascular Endothelium Revealed by in Vivo Phage Display. J. Clin. Invest. July 1998, Vol. 102, pages 430-437, see entire document.	1-14 and 61-63
Y	ARAP, P. et al. Cancer Treatment by Targeted Drug Delivery to Tumor Vasculature in a Mouse Model. Science. January 1998, Vol. 279, pages 377-380, see entire document.	1-14 and 61-63
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:	* ¹ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention * ² document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone * ³ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art * ⁴ document member of the same patent family	
* ¹ document defining the general state of the art which is not considered to be of particular relevance		
* ² earlier document published on or after the international filing date		
* ³ document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
* ⁴ document referring to an oral disclosure, use, exhibition or other means		
* ⁵ document published prior to the international filing date but later than the priority date claimed		
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INTERNATIONAL SEARCH REPORT

International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAJOTTE, D. et al. Membrane Dipeptidase Is The Receptor for a Lung-targeting Peptide Identified by in Vivo Phage Display. J. Biol. Chem. April 1999, Vol. 274, pages 11593-11598, see entire document.	1-14 and 61-63

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08044

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos. 1-14 and 61-68

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/28044A. CLASSIFICATION OF SUBJECT MATTER
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BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This International Search Authority has found 577 inventions claimed in the International Application covered by the claims indicated below:

This application contains the following inventions or groups of inventions, which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-14, 61-63, drawn to a method for identifying a targeting peptide.

Group II, claim(s) 15, drawn to another method for identifying a targeting peptide.

Group III, claim(s) 16-56, drawn to an isolated peptide, a fusion peptide, a composition, a kit comprising the peptide and antibody against the peptide (There are 381 subgroups of inventions within this group because each SEQ ID NOs: 5-395 represents a structural and functional different peptide, which constitutes a separate distinctive invention. Please select one peptide encoded by one sequence to be examined on the merits).

Group IV, claim(s) 37-40, drawn to a gene therapy vector expressing a surface protein comprising peptide (There are 381 subgroups of inventions within this group because each vector carrying a structural and functional distinctive peptide encoding a SEQ ID NO from SEQ ID NOs: 5-395, which constitutes a separate and distinctive inventions. Please select one peptide encoded by one sequence to be examined on the merits).

Group V, claim(s) 41-48, drawn to a method for treating a disease.

Group VI, claim(s) 45-49, drawn to an isolated peptide (There are 245 subgroups of inventions because each SEQ ID NO from SEQ ID NOs: 5-395 represents a structural and functional different peptide, which constitutes a separate distinctive invention. Please select one peptide encoded by one sequence to be examined on the merits).

Group VII, claim(s) 60, drawn to an imaging method.

Group VIII, claim(s) 51-60, drawn to a method for diagnosis.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

1) a plasmid, 2) a cosmid, 3) a yeast, 4) artificial chromosome (YAC), 5) bacterial artificial chromosome (BAC), 6) a virus and 7) a phage.

The following claim(s) are generic: 86. If the group VII is elected, please further select one of the vectors listed in claim 40 to be examined on the merits.

1. This International Searching Authority considers that the international application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-VIII and subgroups of the inventions in groups III, IV and VI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features for studying organ-selective targeting based on in vivo screening of random peptide phage libraries sequence are already known in the art as evidenced by Pasquini et al. (Nature 1998, Vol. 395, pp.264-266. See entire document). Therefore, the unity is lacking among the different groups of the invention.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: different species are directed to the structural and functional different vectors, e.g. the viral vector is very different from the artificial bacterial chromosome in term of the replication property and multiple cloning site as well as insertion capacity. Therefore, they are not so related as a single inventive concept.

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- (71) Applicant (for all designated States except US): BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).
- (72) Inventors; and
(75) Inventors/Applicants (for US only): ARAP, Wadiah [BR/US]; 7171 Buffalo Speedway #328, Houston, TX 77025 (US). PASQUALINI, Renata [BR/US]; 7171 Buffalo Speedway #328, Houston, TX 77025 (US).
- (74) Agents: MALLIE, Michael, J. et al.; Blakely, Sokoloff, Taylor & Zafman LLP, 7th floor, 12400 Wilshire Boulevard, Los Angeles, CA 90025 (US)
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(54) Title: COMPOSITIONS AND METHODS FOR TARGETING PEPTIDES IN HUMANS *IN VIVO*

(57) Abstract: The present invention concerns methods and compositions for identifying human targeting peptides sequences. In preferred embodiments, a primary phage library is administered to a human *in vivo* and phage bearing targeting peptides are collected from selected organs, tissues or cells types. In more preferred embodiments, collected phage are amplified, pooled and administered to a second human for another round of selection. The peptides are of use for targeted delivery of therapeutic agents, including gene therapy vectors. Such targeted delivery may be used for detection, diagnosis or treatment of human diseases. In certain embodiments, the peptide may be attached to an imaging agent and administered to a human to obtain an image or to diagnose a disease state. Also disclosed are a large number of targeting peptide sequences and consensus motifs that are selective for human organs or tissues, obtained by the methods of the present invention.

COMPOSITIONS AND METHODS FOR TARGETING
PEPTIDES IN HUMANS *IN VIVO*

BACKGROUND OF THE INVENTION

This application claims the benefit of U.S. Provisional Patent Application No. 60/231,266 filed September 8, 2000, and U.S. Patent Application No. 09/765,101, filed January 17, 2001. This invention was made with government support under grants DAMD 17-98-1-8041 and 17-98-1-8581 from the U.S. Army and grants 1R01CA78512-01A1, 1R1CA90810-01 and 1R01CA82976-01 from the National Institutes of Health. The government has certain rights in this invention.

1. Field of the Invention

The present invention concerns the fields of molecular medicine and targeted delivery of therapeutic agents. More specifically, the present invention relates to compositions and methods for identification and use of peptides that selectively target organs, tissues or cell types in the human body.

2. Description of Related Art

Therapeutic treatment of many human disease states is limited by the systemic toxicity of the therapeutic agents used. Cancer therapeutic agents in particular exhibit a very low therapeutic index, with rapidly growing normal tissues such as skin and bone marrow affected at concentrations of agent that are not much higher than the concentrations used to kill tumor cells. Treatment of cancer and other organ, tissue or cell type confined disease states would be greatly facilitated by the development of compositions and methods for targeted delivery to a desired organ, tissue or cell type of a therapeutic agent.

Recently, an *in vivo* selection system was developed using phage display libraries to identify organ, tissue or cell type targeting peptides in a mouse model system. Phage display libraries expressing transgenic peptides on the surface of bacteriophage were initially developed to map epitope binding sites of

immunoglobulins (Smith and Scott, 1986, 1993). Such libraries can be generated by inserting random oligonucleotides into cDNAs encoding a phage surface protein, generating collections of phage particles displaying unique peptides in as many as 10^9 permutations. (Pasqualini and Ruoslahti, 1996, Arap et al, 1998a; Arap et al 1998b).

Intravenous administration of phage display libraries to mice was followed by the recovery of phage from individual organs (Pasqualini and Ruoslahti, 1996). Phage were recovered that were capable of selective homing to the vascular beds of different mouse organs, tissues or cell types, based on the specific targeting peptide sequences expressed on the outer surface of the phage (Pasqualini and Ruoslahti, 1996). A variety of organ and tumor-homing peptides have been identified by this method (Rajotte et al., 1998, 1999; Koivunen et al., 1999; Burg et al., 1999; Pasqualini, 1999). Each of those targeting peptides bound to different receptors that were selectively expressed on the vasculature of the mouse target tissue (Pasqualini, 1999; Pasqualini et al., 2000; Folkman, 1995; Folkman 1997). Tumor-homing peptides bound to receptors that were upregulated in the tumor angiogenic vasculature of mice (Brooks et al., 1994; Pasqualini et al., 2000). In addition to identifying individual targeting peptides selective for an organ, tissue or cell type (Pasqualini and Ruoslahti, 1996; Arap et al, 1998a; Koivunen et al., 1999), this system has been used to identify endothelial cell surface markers that are expressed in mice *in vivo* (Rajotte and Ruoslahti, 1999).

Attachment of therapeutic agents to targeting peptides resulted in the selective delivery of the agent to a desired organ, tissue or cell type in the mouse model system. Targeted delivery of chemotherapeutic agents and proapoptotic peptides to receptors located in tumor angiogenic vasculature resulted in a marked increase in therapeutic efficacy and a decrease in systemic toxicity in tumor-bearing mouse models (Arap et al., 1998a, 1998b; Ellerby et al., 1999).

Previous studies with phage display libraries have relied on a mouse model system to identify targeting peptides and their receptors, under the assumption that human targeting peptides are homologous. However, cell surface receptors may have

a very different distribution and function in humans than in mice. Further, the mouse model system has been exploited to characterize targeting peptides for only a handful of specific organs. A need exists in the art for methods and compositions for identification of organ, tissue or cell type selective targeting sequences in humans that can be of clinical use for targeted delivery of therapeutic agents and *in vivo* imaging.

SUMMARY OF THE INVENTION

The present invention solves a long-standing need in the art by providing compositions and methods for the identifying and using targeting peptides that are selective for human organs, tissues or cell types.

Certain embodiments of the present invention concern methods of identifying targeting peptides, comprising injecting a phage display library into the circulation of a human, obtaining a sample of one or more organs, tissues or cell types from said human and identifying one or more targeting peptides from phage present in said organ, tissue or cell type. In preferred embodiments, phage are recovered from an organ, tissue or cell type of a first human subject and further selected by reinjection into the circulation of a second human, followed by obtaining a sample of the same organ, tissue or cell type and identifying targeting peptides. Screening may be performed multiple times in order to obtain targeting phage of sufficient selectivity.

In another preferred embodiment, the human is brain dead or a terminal wean patient. More preferably, the individual is not a candidate organ donor. In other preferred embodiments, one or more primary phage libraries may be amplified *in vitro*, preferably to 10^{14} TU (transforming units) or higher, more preferably with a diversity of 2×10^8 or more different clones, prior to injection into a human subject. In an even more preferred embodiment, targeting peptide sequences are subjected to a custom designed statistical analysis, described in detail in the following Examples, to identify statistically significant targeting peptide sequences.

In yet another preferred embodiment, phage isolated from multiple organs, tissues or cell types from a first human are mixed together and injected into the circulation of a second human, followed by recovery and characterization of phage from the same organs, tissues or cell types of the second human.

In some embodiments, phage collected from an organ, tissue or cell type may be recovered by contacting a homogenate of the organ, tissue with an appropriate host cell, such as *E. coli* K91 kan, and allowing the phage to multiply within the host cell in between rounds of selection. In alternative embodiments, phage may be recovered by direct amplification, such as by PCR™ of the targeting peptide encoding sequences.

In certain embodiments, the targeting peptides of the present invention are of use for the selective delivery of therapeutic agents, including but not limited to gene therapy vectors and fusion proteins, to specific organs, tissues or cell types in human patients. The skilled artisan will realize that the scope of the claimed methods of use include any disease state that can be treated by targeted delivery of a therapeutic agent to a desired organ, tissue or cell type in a human patient. Although such disease states include those where the diseased cells are confined to a specific organ, tissue or cell type, such as non-metastatic cancer, other disease states may be treated by an organ, tissue or cell type-targeting approach.

One embodiment of the present invention concerns isolated peptides of 100 amino acids or less in size, comprising at least 3 contiguous amino acids of a targeting peptide sequence, selected from any of SEQ ID NO:5 through SEQ ID NO:325.

In a preferred embodiment, the isolated peptide is 50 amino acids or less, more preferably 30 amino acids or less, more preferably 20 amino acids or less, more preferably 10 amino acids or less, or even more preferably 5 amino acids or less in size. In other preferred embodiments, the isolated peptide of claim 1 comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 contiguous amino acids of a targeting peptide sequence, selected from any of SEQ ID NO:5 through SEQ ID NO:325.

In certain embodiments, the isolated peptide is attached to a molecule. In preferred embodiments, the attachment is a covalent attachment. In additional embodiments, the molecule is a drug, a chemotherapeutic agent, a radioisotope, a pro-apoptosis agent, an anti-angiogenic agent, a hormone, a cytokine, a growth factor, a cytotoxic agent, a peptide, a protein, an antibiotic, an antibody, a Fab fragment of an antibody, an imaging agent, a survival factor, an anti-apoptotic agent, a hormone antagonist, a virus, a bacterium, a yeast cell, a mammalian cell, a nucleic acid or an antigen. Those molecules are representative only. Molecules within the scope of the present invention include virtually any molecule that may be attached to a targeting peptide and administered to a human. In preferred embodiments, the pro-apoptosis agent is gramicidin, magainin, melittin, defensin, cecropin, (KLAKLAK)₂ (SEQ ID NO:1), (KLAKKLA)₂ (SEQ ID NO:2), (KAAKKAA)₂ (SEQ ID NO:3) or (KLGKKLG)₃ (SEQ ID NO:4). In other preferred embodiments, the anti-angiogenic agent is angiostatin, pigment epithelium-derived factor, angiotensin, laminin peptides, fibronectin peptides, plasminogen activator inhibitors, tissue metalloproteinase inhibitors, interferons, interleukin 12, platelet factor 4, IP-10, Gro- β , thrombospondin, 2-methoxyestradiol, proliferin-related protein, carboxamidotriazole, CM101, Marimastat, pentosan polysulphate, angiopoietin 2 (Regeneron), interferon-alpha, herbimycin A, PNU145156E, 16K prolactin fragment, Linomide, thalidomide, pentoxifylline, genistein, TNP-470, endostatin, paclitaxel, Docetaxel, polyamines, a proteasome inhibitor, a kinase inhibitor, a signaling inhibitor (SU5416 or SU6668, Sugen, South San Francisco, CA), accutin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 or minocycline. In further preferred embodiments, the cytokine is interleukin 1 (IL-1), IL-2, IL-5, IL-10, IL-11, IL-12, IL-18, interferon- γ (IF- γ), IF- α , IF- β , tumor necrosis factor- α (TNF- α), or GM-CSF (granulocyte macrophage colony stimulating factor). Such examples are representative only and are not intended to exclude other pro-apoptosis agents, anti-angiogenic agents or cytokines known in the art.

In other embodiments, the isolated peptide is attached to a macromolecular complex. In preferred embodiments, the attachment is a covalent attachment. In other preferred embodiments, the macromolecular complex is a virus, a bacteriophage, a bacterium, a liposome, a microparticle, a magnetic bead, a cell, a yeast cell or a microdevice. These are representative examples only. Macromolecular complexes within the scope of the present invention include virtually any macromolecular complex that may be attached to a targeting peptide and administered to a human. In other preferred embodiments, the isolated peptide is attached to a eukaryotic expression vector, more preferably a gene therapy vector.

In another embodiment, the isolated peptide is attached to a solid support, preferably magnetic beads, Sepharose beads, agarose beads, a nitrocellulose membrane, a nylon membrane, a column chromatography matrix, a high performance liquid chromatography (HPLC) matrix or a fast performance liquid chromatography (FPLC) matrix.

Additional embodiments of the present invention concern fusion proteins comprising at least 3 contiguous amino acids of a sequence selected from any of SEQ ID NO:5 through SEQ ID NO:325.

Certain other embodiments concern compositions comprising the claimed isolated peptides or fusion proteins in a pharmaceutically acceptable carrier. Further embodiments concern kits comprising the claimed isolated peptides or fusion proteins in one or more containers.

Other embodiments concern methods of targeted delivery comprising selecting a targeting peptide for a desired organ, tissue or cell type, attaching said targeting peptide to a molecule, macromolecular complex or gene therapy vector, and providing said peptide attached to said molecule, complex or vector to a human. Preferably, the targeting peptide is selected to include at least 3 contiguous amino acids from any of SEQ ID NO:5 through SEQ ID NO:325. In certain preferred embodiments, the organ, tissue or cell type is bone marrow, skin, skeletal muscle, prostate or adipose tissue. In

other preferred embodiments, the molecule attached to the targeting peptide is a chemotherapeutic agent, an antigen or an imaging agent. The skilled artisan will realize that within the scope of the present invention any human organ, tissue or cell type can be targeted for delivery, using targeting peptides attached to any molecule, macromolecular complex or gene therapy vector.

Other embodiments of the present invention concern isolated nucleic acids of 300 nucleotides or less in size, encoding a targeting peptide. In preferred embodiments, the isolated nucleic acid is 250, 225, 200, 175, 150, 125, 100, 75, 50, 40, 30, 20 or even 10 nucleotides or less in size. In other preferred embodiments, the isolated nucleic acid is incorporated into a eukaryotic or a prokaryotic expression vector. In even more preferred embodiments, the vector is a plasmid, a cosmid, a yeast artificial chromosome (YAC), a bacterial artificial chromosome (BAC), a virus or a bacteriophage. In other preferred embodiments, the isolated nucleic acid is operatively linked to a leader sequence that localizes the expressed peptide to the extracellular surface of a host cell.

Additional embodiments of the present invention concern methods of treating a disease state comprising selecting a targeting peptide that targets cells associated with the disease state, attaching one or more molecules effective to treat the disease state to the peptide, and administering the peptide to a human with the disease state. Preferably, the targeting peptide includes at least three contiguous amino acids selected from any of SEQ ID NO:5 through SEQ ID NO:325. In preferred embodiments the disease state is diabetes mellitus, inflammatory disease, arthritis, atherosclerosis, cancer, autoimmune disease, bacterial infection, viral infection, cardiovascular disease, degenerative disease or macular degeneration.

Another embodiment of the present invention concerns compositions and methods of use of tumor targeting peptides against human cancers. Human tumor targeting peptides identified by the methods disclosed in the instant application may be attached to therapeutic agents, including but not limited to molecules or macromolecular assemblages and administered to a patient with cancer, providing for

increased efficacy and decreased systemic toxicity of the therapeutic agent. Therapeutic agents within the scope of the present invention include but are not limited to chemotherapeutic agents, radioisotopes, pro-apoptosis agents, cytotoxic agents, cytostatic agents and gene therapy vectors. Targeted delivery of such therapeutic agents to human tumors provides a significant improvement over the prior art for increasing the delivery of the agent to the tumor, while decreasing the inadvertent delivery of the agent to normal organs and tissues of the patient. In a preferred embodiment, the tumor targeting peptide is incorporated into the capsule of a phage gene therapy vector to target delivery of the phage to angiogenic endothelial cells in tumor blood vessels.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A. *In vivo* phage display screening for peptides that home to human tissues through the systemic circulation. Schematic flowchart of the study.

FIG. 1B. Phage recovery from various human tissues *in vivo*. Tissue samples were processed and phage recovered as described in Example 3. Shown are means \pm standard errors of the mean of phage transducing units (TU) per gram of tissue obtained from each biopsy site.

FIG. 2A. Identification of extended homing motifs with the ClustalW program (European Molecular Biology laboratory; EMBL). Peptide sequences containing selected tripeptides (Table 3) enriched in each single tissue were aligned in clusters to obtain longer motifs shared between different peptides from each cluster. The software registers sequence identities and similarities among multiple peptide sequences and aligns the sequences by placing peptides with the most identity/similarity next to one another. The original and extended peptide motifs are shown highlighted in bold.

FIG. 2B. Identification of extended homing motifs with the ClustalW program (European Molecular Biology laboratory; EMBL). Peptide sequences containing selected tripeptides (Table 3) enriched in multiple tissues were aligned in clusters to obtain longer motifs shared between different peptides from each cluster. The software registers sequence identities and similarities among multiple peptide sequences and aligns the sequences by placing peptides with the most identity/similarity next to one another. The original and extended peptide motifs are shown highlighted in bold.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

As used herein in the specification, "a" or "an" may mean one or more. As used herein in the claim(s), in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more of an item.

A "targeting peptide" is a peptide comprising a contiguous sequence of amino acids, that is characterized by selective localization to a human organ, tissue or cell type. Selective localization may be determined, for example, by methods disclosed below, wherein the putative targeting peptide sequence is incorporated into a protein that is displayed on the outer surface of a phage. Administration to a human of a library of such phage that have been genetically engineered to express a multitude of such targeting peptides of different amino acid sequence is followed collection of one or more organs, tissues or cell types from the human and identification of phage found in that organ, tissue or cell type. A phage expressing a targeting peptide sequence is considered to be selectively localized to a tissue or organ if it exhibits greater binding in that tissue or organ compared to a control tissue or organ. Preferably, selective localization of a targeting peptide will result in a two-fold or higher enrichment of the phage in the target organ, tissue or cell type, compared to a control organ, tissue or cell type. Alternatively, a phage expressing a targeting peptide sequence that exhibits selective localization preferably shows an increased enrichment in the target organ compared to a control organ when phage recovered from the target organ are reinjected

into a second host for another round of screening. Further enrichment may be exhibited following a third round of screening. Another alternative method to determine selective localization is that phage expressing the putative target peptide preferably exhibit a two-fold, more preferably a three-fold or higher enrichment in the target organ compared to control phage that express a non-specific peptide or that have not been genetically engineered to express any putative target peptides. Another method to determine selective localization is that localization to the target organ of phage expressing the target peptide is at least partially blocked by the co-administration of a synthetic peptide containing the target peptide sequence. "Targeting peptide" and "homing peptide" are used synonymously herein.

A "phage display library" means a collection of phage that have been genetically engineered to express a set of putative targeting peptides on their outer surface. In preferred embodiments, DNA sequences encoding the putative targeting peptides are inserted in frame into a gene encoding a phage capsule protein. In other preferred embodiments, the putative targeting peptide sequences are in part random mixtures of all twenty amino acids and in part non-random. In certain preferred embodiments the putative targeting peptides of the phage display library exhibit one or more cysteine residues at fixed locations within the targeting peptide sequence.

A "macromolecular complex" refers to a collection of molecules that may be random, ordered or partially ordered in their arrangement. The term encompasses biological organisms such as bacteriophage, viruses, bacteria, unicellular pathogenic organisms, multicellular pathogenic organisms and prokaryotic or eukaryotic cells. The term also encompasses non-living assemblages of molecules, such as liposomes, microcapsules, microparticles, magnetic beads and microdevices. The only requirement is that the complex contains more than one molecule. The molecules may be identical, or may differ from each other.

A "receptor" for a targeting peptide includes but is not limited to any molecule or complex of molecules that binds to a targeting peptide. Non-limiting examples of

receptors include peptides, proteins, glycoproteins, lipoproteins, epitopes, lipids, carbohydrates, multi-molecular structures, a specific conformation of one or more molecules and a morphoanatomic entity. In preferred embodiments, a "receptor" is a naturally occurring molecule or complex of molecules that is present on the luminal surface of cells forming blood vessels within a target organ, tissue or cell type.

A "subject" refers generally to a mammal. In certain preferred embodiments, the subject is a mouse or rabbit. In even more preferred embodiments, the subject is a human.

Phage Display

The methods described herein for identification of targeting peptides involve the *in vivo* administration of phage display libraries. Various methods of phage display and methods for producing diverse populations of peptides are well known in the art. For example, U.S. Pat. Nos. 5,223,409; 5,622,699 and 6,068,829, each of which is incorporated herein by reference, disclose methods for preparing a phage library. The phage display technique involves genetically manipulating bacteriophage so that small peptides can be expressed on their surface (Smith *et al.*, 1985, 1993). The potential range of applications for this technique is quite broad, and the past decade has seen considerable progress in the construction of phage-displayed peptide libraries and in the development of screening methods in which the libraries are used to isolate peptide ligands. For example, the use of peptide libraries has made it possible to characterize interacting sites and receptor-ligand binding motifs within many proteins, such as antibodies involved in inflammatory reactions or integrins that mediate cellular adherence. This method has also been used to identify novel peptide ligands that serve as leads to the development of peptidomimetic drugs or imaging agents (Arap *et al.*, 1998a). In addition to peptides, larger protein domains such as single-chain antibodies can also be displayed on the surface of phage particles (Arap *et al.*, 1998a).

Amino acid sequences for a targeting given organ, tissue or cell type can be isolated by "biopanning" (Pasqualini and Ruoslahti, 1996; Pasqualini, 1999). In brief, a

library of phage containing putative targeting peptides is administered to an animal or human and samples of organs, tissues or cell types containing phage are collected. In preferred embodiments utilizing filamentous phage, the phage may be propagated *in vitro* between rounds of biopanning in pilus-positive bacteria. The bacteria are not lysed by the phage but rather secrete multiple copies of phage that display a particular insert. Phage that bind to a target molecule can be eluted from the target organ, tissue or cell type and then amplified by growing them in host bacteria. If desired, the amplified phage can be administered to a human host and samples of organs, tissues or cell types again collected. Multiple rounds of biopanning may be performed until a population of selective binders is obtained. The amino acid sequence of the peptides is determined by sequencing the DNA corresponding to the targeting peptide insert in the phage genome. The identified targeting peptide can then be produced as a synthetic peptide by standard protein chemistry techniques (Arap *et al.*, 1998a, Smith *et al.*, 1985). This approach allows circulating targeting peptides to be detected in an unbiased functional assay, without any preconceived notions about the nature of their target. Once a candidate target is identified as the receptor of a targeting peptide, it can be isolated, purified and cloned by using standard biochemical methods (Pasqualini, 1999; Rajotte and Ruoslahti, 1999).

Choice of phage display system.

Previous *in vivo* selection studies performed in mice preferentially employed libraries of random peptides expressed as fusion proteins with the gene III capsule protein in the FUSE5 vector (Pasqualini and Ruoslahti, 1996). The number and diversity of individual clones present in a given library is a significant factor for the success of *in vivo* selection. It is preferred to use primary libraries, which are less likely to have an over-representation of defective phage clones (Koivunen *et al.*, 1999). The preparation of a library may be amplified to between 10^8 - 10^9 transducing units (T.U.)/ml. Preferably, 10^{14} TU or more are prepared from a primary library for injection into human subjects. In certain embodiments, a bulk amplification strategy is applied between each round of selection.

Phage libraries displaying linear, cyclic, or double cyclic peptides may be used within the scope of the present invention. However, phage libraries displaying 3 to 10 random residues in a cyclic insert (CX₃₋₁₀C) are preferred, since single cyclic peptides tend to have a higher affinity for the target organ than linear peptides. Libraries displaying double-cyclic peptides (such as CX₃C X₃CX₃C; Rajotte *et al.*, 1998) have been successfully used. However, the production of the cognate synthetic peptides, although possible, can be complex due to the multiple conformers with different disulfide bridge arrangements.

Identification of homing peptides and receptors by in vivo phage display in mice.

In vivo selection of peptides from phage-display peptide libraries administered to mice has been used to identify targeting peptides selective for normal mouse brain, kidney, lung, skin, pancreas, retina, intestine, uterus, prostate, and adrenal gland (Pasqualini and Ruoslahti, 1996; Pasqualini, 1999; Rajotte *et al.*, 1998). These results show that the vascular endothelium of normal organs is sufficiently heterogeneous to allow differential targeting with peptide probes (Pasqualini and Ruoslahti, 1996; Rajotte *et al.*, 1998). A means of identifying peptides that home to the angiogenic vasculature of tumors has been devised, as described below. A panel of peptide motifs that target the blood vessels of tumor xenografts in nude mice has been assembled (Arap *et al.*, 1998a; reviewed in Pasqualini, 1999). These motifs include the sequences RGD-4C, NGR, and GSL. The RGD-4C peptide has previously been identified as selectively binding α_v integrins and has been shown to home to the vasculature of tumor xenografts in nude mice (Arap *et al.*, 1998a, 1998b; Pasqualini *et al.*, 1997).

The receptors for the tumor homing RGD4C targeting peptide has been identified as α_v integrins (Pasqualini *et al.*, 1997). The α_v integrins play an important role in angiogenesis. The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are absent or expressed at low levels in normal endothelial cells but are induced in angiogenic vasculature of tumors (Brooks *et al.*, 1994; Hammes *et al.*, 1996). Aminopeptidase N/CD13 has recently been

identified as an angiogenic receptor for the NGR motif (Burg *et al.*, 1999). Aminopeptidase N/CD13 is strongly expressed not only in the angiogenic blood vessels of prostate cancer in TRAMP mice but also in the normal epithelial prostate tissue.

Tumor-homing phage co-localize with their receptors in the angiogenic vasculature of tumors but not in non-angiogenic blood vessels in normal tissues (Arap *et al.*, 1998b). Immunohistochemical evidence shows that vascular targeting phage bind to human tumor blood vessels in tissue sections (Pasqualini *et al.*, 2000) but not to normal blood vessels. A negative control phage with no insert (fd phage) did not bind to normal or tumor tissue sections. The expression of the angiogenic receptors was evaluated in cell lines, in non-proliferating blood vessels and in activated blood vessels of tumors and other angiogenic tissues such as corpus luteum. Flow cytometry and immunohistochemistry showed that these receptors are expressed in a number of tumor cells and in activated HUVECs (data not shown). The angiogenic receptors were not detected in the vasculature of normal organs of mouse or human tissues.

The distribution of these receptors was analyzed by immunohistochemistry in tumor cells, tumor vasculature, and normal vasculature. Alpha v integrins, CD13, aminopeptidase A, NG2, and MMP-2/MMP-9 - the known receptors in tumor blood vessels - are specifically expressed in angiogenic endothelial cells and pericytes of both human and murine origin. Angiogenic neovasculature expresses markers that are either expressed at very low levels or not at all in non-proliferating endothelial cells (not shown).

The markers of angiogenic endothelium include receptors for vascular growth factors, such as specific subtypes of VEGF and basic FGF receptors, and α_v integrins, among many others (Mustonen and Alitalo, 1995). Thus far, identification and isolation of novel molecules characteristic of angiogenic vasculature has been slow, mainly because endothelial cells undergo dramatic phenotypic changes when grown in culture (Watson *et al.*, 1995).

Many of these tumor vascular markers are proteases and some of the markers also serve as viral receptors. Alpha v integrins are receptors for adenoviruses (Wickham *et al.*, 1997c) and CD13 is a receptor for coronaviruses (Look *et al.*, 1989). MMP-2 and MMP-9 are receptors for echoviruses (Koivunen *et al.*, 1999). Aminopeptidase A also appears to be a viral receptor. Bacteriophage may use the same cellular receptors as eukaryotic viruses. These findings suggest that receptors isolated by *in vivo* phage display will have cell internalization capability, a key feature for utilizing the identified peptide motifs as targeted gene therapy carriers.

Targeted delivery

Peptides that home to tumor vasculature have been coupled to cytotoxic drugs or proapoptotic peptides to yield compounds that were more effective and less toxic than the parental compounds in experimental models of mice bearing tumor xenografts (Arap *et al.*, 1998a; Ellerby *et al.*, 1999). The insertion of the RGD-4C peptide into a surface protein of an adenovirus has produced an adenoviral vector that may be used for tumor targeted gene therapy (Arap *et al.*, 1998b).

BRASIL

In preferred embodiments, separation of phage bound to the cells of a target organ, tissue or cell type from unbound phage is achieved using the BRASIL technique (Provisional Patent Application No. 60/231,266 filed September 8, 2000; U.S. Patent Application entitled, "Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL)" by Arap, Pasqualini and Giordano, filed concurrently herewith, incorporated herein by reference in its entirety). In BRASIL (Biopanning and Rapid Analysis of Soluble Interactive Ligands), an organ, tissue or cell type is gently separated into cells or small clumps of cells that are suspended in an aqueous phase. The aqueous phase is layered over an organic phase of appropriate density and centrifuged. Cells attached to bound phage are pelleted at the bottom of the centrifuge tube, while unbound phage remain in the aqueous phase. This allows a more efficient separation of bound from unbound phage, while maintaining the binding interaction between phage

and cell. BRASIL may be performed in an *in vivo* protocol, in which organs, tissues or cell types are exposed to a phage display library by intravenous administration, or by an *ex vivo* protocol, where the cells are exposed to the phage library in the aqueous phase before centrifugation.

In certain embodiments, a subtraction protocol is used with BRASIL or other screening protocols to further reduce background phage binding. The purpose of subtraction is to remove phage from the library that bind to cells other than the cell of interest, or that bind to inactivated cells. In alternative embodiments, the phage library may be screened against a control cell line, tissue or organ sample that is not the targeted cell, tissue or organ. After subtraction the library may be screened against the cell, tissue or organ of interest. In another alternative embodiment, an unstimulated, quiescent cell line, tissue or organ may be screened against the library and binding phage removed. The cell line, tissue or organ is then activated, for example by administration of a hormone, growth factor, cytokine or chemokine and the activated cell line screened against the subtracted phage library.

Other methods of subtraction protocols are known and may be used in the practice of the present invention, for example as disclosed in U.S. Patent Nos. 5,840,841, 5,705,610, 5,670,312 and 5,492,807, incorporated herein by reference.

Preparation of large scale primary libraries

In preferred embodiments, primary phage libraries are amplified before injection into a human subject. A phage library is prepared by ligating targeting peptide encoding sequences into a phage vector, such as FUSE5. The vector is transformed into pilus negative host *E. coli* such as strain MC1061. The bacteria are grown overnight and then aliquots are frozen to provide stock for library production. Use of pilus negative bacteria avoids the bias in libraries that arises from differential infection of pilus positive bacteria by different targeting peptide sequences.

To freeze, bacteria are pelleted from two thirds of a primary library culture (5 liters) at 4000 x g for 10 min. Bacteria are resuspended and washed twice with 500 ml of 10% glycerol in water, then frozen in an ethanol/dry ice bath and stored at -80°C.

For amplification, 1.5 ml of frozen bacteria are inoculated into 5 liters of LB medium with 20 µg/ml tetracycline and grown overnight. Thirty minutes after inoculation, a serial dilution is plated on LB/tet plates to verify the viability of the culture. If the number of viable bacteria is less than 5-10 times the number of individual clones in the library ($1-2 \times 10^6$) the culture is discarded.

After growing the bacterial culture overnight, phage are precipitated. About 1/4 to 1/3 of the bacterial culture is kept growing overnight in 5 liters of fresh medium and the cycle is repeated up to 5 times. Phage are pooled from all cycles and used for injection into human subjects.

Humans

The methods used for phage display biopanning in the mouse model system require substantial improvements for use with humans. A preferred example of the phage display protocol used for human patients, disclosing these improvements, is described in detail in Examples 1 and 2 below.

In general, humans suitable for use with phage display are either brain dead or terminal wean patients. The amount of phage library (preferably primary library) required for administration must be significantly increased, preferably to 10^{14} TU or higher, preferably administered intravenously in approximately 200 ml of Ringer lactate solution over about a 10 minute period.

The amount of phage required for use in humans has required improvement of the mouse protocol, increasing the amount of phage available for injection by five orders of magnitude. To produce such large phage libraries, the transformed bacterial pellets recovered from up to 500 to 1000 transformations are amplified up to 10 times in the bacterial host, recovering the phage from each round of amplification and adding LB Tet medium to the bacterial pellet for collection of additional phage. The phage

inserts remain stable under these conditions and phage may be pooled to form the large phage display library required for humans.

Samples of various organs and tissues are collected starting approximately 15 minutes after injection of the phage library. Samples are processed as described below and phage collected from each organ, tissue or cell type of interest for DNA sequencing to determine the amino acid sequences of targeting peptides.

With humans, the opportunities for enrichment by multiple rounds of biopanning are severely restricted, compared to the mouse model system. A substantial improvement in the biopanning technique involves polyorgan targeting.

Polyorgan targeting

In the standard protocol for phage display biopanning, phage from a single organ are collected, amplified and injected into a new host, where tissue from the same organ is collected for phage rescue and a new round of biopanning. This protocol is feasible in animal subjects. However, the limited availability and expense of processing samples from humans requires an improvement in the protocol.

As disclosed in a preferred embodiment, described in Example 3 below, it is possible to pool phage collected from multiple organs after a first round of biopanning and inject the pooled sample into a new subject, where each of the multiple organs may be collected again for phage rescue. The polyorgan targeting protocol may be repeated for as many rounds of biopanning as desired. In this manner, it is possible to significantly reduce the number of subjects required for isolation of targeting peptides for multiple organs, while still achieving substantial enrichment of the organ-homing phage.

In preferred embodiments, phage are recovered from human organs, tissues or cell types after injection of a phage display library into a human subject. In certain embodiments, phage may be recovered by exposing a sample of the organ, tissue or cell type to a pilus positive bacterium, such as *E. coli* K91. In alternative embodiments,

phage may be recovered by amplifying the phage inserts, ligating the inserts to phage DNA and producing new phage from the ligated DNA.

Proteins and Peptides

In certain embodiments, the present invention concerns novel compositions comprising at least one protein or peptide. As used herein, a protein or peptide generally refers, but is not limited to, a protein of greater than about 200 amino acids, up to a full length sequence translated from a gene; a polypeptide of greater than about 100 amino acids; and/or a peptide of from about 3 to about 100 amino acids. For convenience, the terms "protein," "polypeptide" and "peptide are used interchangeably herein.

In certain embodiments the size of the at least one protein or peptide may comprise, but is not limited to, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 475, about 500, about 525, about 550, about 575, about 600, about 625, about 650, about 675, about 700, about 725, about 750, about 775, about 800, about 825, about 850, about 875, about 900, about 925, about 950, about 975, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1750, about 2000, about 2250, about 2500 or greater amino acid residues.

As used herein, an "amino acid residue" refers to any naturally occurring amino acid, any amino acid derivative or any amino acid mimic known in the art. In certain embodiments, the residues of the protein or peptide are sequential, without any non-

amino acid interrupting the sequence of amino acid residues. In other embodiments, the sequence may comprise one or more non-amino acid moieties. In particular embodiments, the sequence of residues of the protein or peptide may be interrupted by one or more non-amino acid moieties.

Accordingly, the term "protein or peptide" encompasses amino acid sequences comprising at least one of the 20 common amino acids found in naturally occurring proteins, or at least one modified or unusual amino acid, including but not limited to those shown on Table 1 below.

TABLE 1
Modified and Unusual Amino Acids

Abbr.	Amino Acid	Abbr.	Amino Acid
Aad	2-Aminoadipic acid	EtAsn	N-Ethylasparagine
Baad	3- Amino adipic acid	Hyl	Hydroxylysine
Bala	β -alanine, β -Amino-propionic acid	AHyl	allo-Hydroxylysine
Abu	2-Aminobutyric acid	3Hyp	3-Hydroxyproline
4Abu	4- Aminobutyric acid, piperidinic acid	4Hyp	4-Hydroxyproline
Acp	6-Aminocaproic acid	Ide	Isodesmosine
Ahe	2-Aminoheptanoic acid	Alle	allo-Isoleucine
Aib	2-Aminoisobutyric acid	MeGly	N-Methylglycine, sarcosine
Baib	3-Aminoisobutyric acid	Melle	N-Methylisoleucine
Apm	2-Aminopimelic acid	MeLys	6-N-Methyllysine
Dbu	2,4-Diaminobutyric acid	MeVal	N-Methylvaline
Des	Desmosine	Nva	Norvaline
Dpm	2,2'-Diaminopimelic acid	Nle	Norleucine
Dpr	2,3-Diaminopropionic acid	Orn	Ornithine
EtGly	N-Ethylglycine		

Proteins or peptides may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteins or peptides from natural sources, or the chemical synthesis of proteins or peptides. The nucleotide and protein, polypeptide and peptide sequences corresponding to various genes have been previously disclosed, and may be found at computerized databases known to those of ordinary skill in the art. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (<http://www.ncbi.nlm.nih.gov/>). The coding regions for known genes may be amplified and/or expressed using the techniques disclosed herein or as would be known to those of ordinary skill in the art. Alternatively, various commercial preparations of proteins, polypeptides and peptides are known to those of skill in the art.

Peptide mimetics

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, "Peptide Turn Mimetics" in *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, Eds., Chapman and Hall, New York (1993), incorporated herein by reference. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These principles may be used to engineer second generation molecules having many of the natural properties of the targeting peptides disclosed herein, but with altered and even improved characteristics.

Fusion proteins

Other embodiments of the present invention concern fusion proteins. These molecules generally have all or a substantial portion of a targeting peptide, linked at the N- or C-terminus, to all or a portion of a second polypeptide or protein. For example, fusions may employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions. In preferred embodiments, the fusion proteins of the instant invention comprise a targeting peptide linked to a therapeutic protein or peptide. Examples of proteins or peptides that may be incorporated into a fusion protein include cytostatic proteins, cytotoxic proteins, pro-apoptosis agents, anti-angiogenic agents, hormones, cytokines, growth factors, peptide drugs, antibodies, Fab fragments antibodies, antigens, receptor proteins, enzymes, lectins, MHC proteins, cell adhesion proteins and binding proteins. These examples are not meant to be limiting and it is contemplated that within the scope of the present invention virtually any protein or peptide could be incorporated into a fusion protein comprising a targeting peptide. Methods of generating fusion proteins are well known to those of skill in the art. Such proteins can be produced, for example, by chemical attachment using bifunctional cross-linking reagents, by *de novo* synthesis of the complete fusion protein, or by attachment of a DNA sequence encoding the targeting peptide to a DNA sequence encoding the second peptide or protein, followed by expression of the intact fusion protein.

Protein purification

In certain embodiments a protein or peptide may be isolated or purified. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the homogenization and crude fractionation of the cells, tissue or

organ to polypeptide and non-polypeptide fractions. The protein or polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, gel exclusion chromatography, polyacrylamide gel electrophoresis, affinity chromatography, immunoaffinity chromatography and isoelectric focusing. An example of receptor protein purification by affinity chromatography is disclosed in U.S. Patent No. 5,206,347, the entire text of which is incorporated herein by reference. A particularly efficient method of purifying peptides is fast protein liquid chromatography (FPLC) or even HPLC.

A purified protein or peptide is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. An isolated or purified protein or peptide, therefore, also refers to a protein or peptide free from the environment in which it may naturally occur. Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide are known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity therein, assessed by a "fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the

particular assay technique chosen to follow the purification, and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification are well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like, or by heat denaturation, followed by: centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of these and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule to which it can specifically bind to. This is a receptor-ligand type of interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (e.g., altered pH, ionic strength, temperature, etc.). The matrix should be a substance

that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand.

Synthetic Peptides

Because of their relatively small size, the targeting peptides of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, (1984); Tam *et al.*, (1983); Merrifield, (1986); and Barany and Merrifield (1979), each incorporated herein by reference. Short peptide sequences, usually from about 6 up to about 35 to 50 amino acids, can be readily synthesized by such methods. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression.

Antibodies

In certain embodiments, it may be desirable to make antibodies against the identified targeting peptides or their receptors. The appropriate targeting peptide or receptor, or portions thereof, may be coupled, bonded, bound, conjugated, or chemically-linked to one or more agents via linkers, polylinkers, or derivatized amino acids. This may be performed such that a bispecific or multivalent composition or vaccine is produced. It is further envisioned that the methods used in the preparation of these compositions are familiar to those of skill in the art and should be suitable for administration to humans, *i.e.*, pharmaceutically acceptable. Preferred agents are the carriers are keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA).

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, F(ab')₂, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. Techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, e.g., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

Cytokines and chemokines

In certain embodiments, it may be desirable to couple specific bioactive agents to one or more targeting peptides for targeted delivery to an organ, tissue or cell type. Such agents include, but are not limited to, cytokines, chemokines, pro-apoptosis factors and anti-angiogenic factors. The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, growth factors and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; prostaglandin, fibroblast growth factor; prolactin; placental lactogen, OB protein; tumor necrosis factor- α and - β .; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- β .; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- β .; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, LIF, G-CSF, GM-CSF, M-CSF, EPO, kit-ligand

or FLT-3, angiostatin, thrombospondin, endostatin, tumor necrosis factor and LT. As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

Chemokines generally act as chemoattractants to recruit immune effector cells to the site of chemokine expression. It may be advantageous to express a particular chemokine gene in combination with, for example, a cytokine gene, to enhance the recruitment of other immune system components to the site of treatment. Chemokines include, but are not limited to, RANTES, MCAF, MIP1-alpha, MIP1-Beta, and IP-10. The skilled artisan will recognize that certain cytokines are also known to have chemoattractant effects and could also be classified under the term chemokines.

Imaging agents and radioisotopes

In certain embodiments, the claimed peptides or proteins of the present invention may be attached to imaging agents of use for imaging and diagnosis of various diseased organs, tissues or cell types. Many appropriate imaging agents are known in the art, as are methods for their attachment to proteins or peptides (see, e.g., U.S. patents 5,021,236 and 4,472,509, both incorporated herein by reference). Certain attachment methods involve the use of a metal chelate complex employing, for example, an organic chelating agent such as DTPA attached to the protein or peptide (U.S. Patent 4,472,509). Proteins or peptides also may be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate.

Non-limiting examples of paramagnetic ions of potential use as imaging agents include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being particularly preferred. Ions useful in other contexts, such as X-ray imaging,

include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III).

Radioisotopes of potential use as imaging or therapeutic agents include astatine²¹¹, ¹⁴carbon, ⁵¹chromium, ³⁶chlorine, ⁵⁷cobalt, ⁵⁸cobalt, copper⁶⁷, ¹⁵²Eu, gallium⁶⁷, ³hydrogen, iodine¹²³, iodine¹²⁵, iodine¹³¹, indium¹¹¹, ⁵⁹iron, ³²phosphorus, rhenium¹⁸⁶, rhenium¹⁸⁸, ⁷⁵selenium, ³⁵sulphur, technetium^{99m} and yttrium⁹⁰. ¹²⁵I is often being preferred for use in certain embodiments, and technetium^{99m} and indium¹¹¹ are also often preferred due to their low energy and suitability for long range detection.

Radioactively labeled proteins or peptides of the present invention may be produced according to well-known methods in the art. For instance, they can be iodinated by contact with sodium or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Proteins or peptides according to the invention may be labeled with technetium-^{99m} by ligand exchange process, for example, by reducing pertechnetate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the peptide to this column or by direct labeling techniques, *e.g.*, by incubating pertechnetate, a reducing agent such as SnCl_2 , a buffer solution such as sodium-potassium phthalate solution, and the peptide. Intermediary functional groups which are often used to bind radioisotopes which exist as metallic ions to peptides are diethylenetriaminedipentaacetic acid (DTPA) and ethylene diaminetetracetic acid (EDTA). Also contemplated for use are fluorescent labels, including rhodamine, fluorescein isothiocyanate and renographin.

In certain embodiments, the claimed proteins or peptides may be linked to a secondary binding ligand or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of suitable enzymes include urcase, alkaline phosphatase, (horseradish) hydrogen peroxidase and glucose oxidase. Preferred secondary binding ligands are biotin and avidin or streptavidin compounds. The use of such labels is well known to those of skill in the art in light and

is described, for example, in U.S. Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241; each incorporated herein by reference.

Cross-linkers

Bifunctional cross-linking reagents have been extensively used for a variety of purposes including preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites, and structural studies. Homobifunctional reagents that carry two identical functional groups proved to be highly efficient in inducing cross-linking between identical and different macromolecules or subunits of a macromolecule, and linking of polypeptide ligands to their specific binding sites. Heterobifunctional reagents contain two different functional groups. By taking advantage of the differential reactivities of the two different functional groups, cross-linking can be controlled both selectively and sequentially. The bifunctional cross-linking reagents can be divided according to the specificity of their functional groups, *e.g.*, amino, sulfhydryl, guanidino, indole, carboxyl specific groups. Of these, reagents directed to free amino groups have become especially popular because of their commercial availability, ease of synthesis and the mild reaction conditions under which they can be applied. A majority of heterobifunctional cross-linking reagents contains a primary amine-reactive group and a thiol-reactive group.

Exemplary methods for cross-linking ligands to liposomes are described in U.S. Patent 5,603,872 and U.S. Patent 5,401,511, each specifically incorporated herein by reference in its entirety). Various ligands can be covalently bound to liposomal surfaces through the cross-linking of amine residues. Liposomes, in particular, multilamellar vesicles (MLV) or unilamellar vesicles such as microemulsified liposomes (MEL) and large unilamellar liposomes (LUVET), each containing phosphatidylethanolamine (PE), have been prepared by established procedures. The inclusion of PE in the liposome provides an active functional residue, a primary amine, on the liposomal surface for cross-linking purposes. Ligands such as epidermal growth factor (EGF) have been successfully linked with PE-liposomes. Ligands are bound

covalently to discrete sites on the liposome surfaces. The number and surface density of these sites are dictated by the liposome formulation and the liposome type. The liposomal surfaces may also have sites for non-covalent association. To form covalent conjugates of ligands and liposomes, cross-linking reagents have been studied for effectiveness and biocompatibility. Cross-linking reagents include glutaraldehyde (GAD), bifunctional oxirane (OXR), ethylene glycol diglycidyl ether (EGDE), and a water soluble carbodiimide, preferably 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Through the complex chemistry of cross-linking, linkage of the amine residues of the recognizing substance and liposomes is established.

In another example, heterobifunctional cross-linking reagents and methods of using the cross-linking reagents are described (U.S. Patent 5,889,155, specifically incorporated herein by reference in its entirety). The cross-linking reagents combine a nucleophilic hydrazide residue with an electrophilic maleimide residue, allowing coupling in one example, of aldehydes to free thiols. The cross-linking reagent can be modified to cross-link various functional groups.

Nucleic Acids

Nucleic acids according to the present invention may encode a targeting peptide, a receptor protein or a fusion protein. The nucleic acid may be derived from genomic DNA, complementary DNA (cDNA) or synthetic DNA. Where incorporation into an expression vector is desired, the nucleic acid may also comprise a natural intron or an intron derived from another gene. Such engineered molecules are sometime referred to as "mini-genes."

A "nucleic acid" as used herein includes single-stranded and double-stranded molecules, as well as DNA, RNA, chemically modified nucleic acids and nucleic acid analogs. It is contemplated that a nucleic acid within the scope of the present invention may be of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71,

72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 475, about 500, about 525, about 550, about 575, about 600, about 625, about 650, about 675, about 700, about 725, about 750, about 775, about 800, about 825, about 850, about 875, about 900, about 925, about 950, about 975, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1750, about 2000, about 2250, about 2500 or greater nucleotide residues in length.

It is contemplated that targeting peptides, fusion proteins and receptors may be encoded by any nucleic acid sequence that encodes the appropriate amino acid sequence. The design and production of nucleic acids encoding a desired amino acid sequence is well known to those of skill in the art, using standardized codon tables (see Table 2 below). In preferred embodiments, the codons selected for encoding each amino acid may be modified to optimize expression of the nucleic acid in the host cell of interest. Codon preferences for various species of host cell are well known in the art.

TABLE 2

Amino Acid			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU

Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

In addition to nucleic acids encoding the desired targeting peptide, fusion protein or receptor amino acid sequence, the present invention encompasses complementary nucleic acids that hybridize under high stringency conditions with such coding nucleic acid sequences. High stringency conditions for nucleic acid hybridization are well known in the art. For example, conditions may comprise low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the

particular nucleic acid(s), the length and nucleotide content of the target sequence(s), the charge composition of the nucleic acid(s), and to the presence or concentration of formamide, tetramethylammonium chloride or other solvent(s) in a hybridization mixture.

Vectors for Cloning, Gene Transfer and Expression

In certain embodiments expression vectors are employed to express the targeting peptide or fusion protein, which can then be purified and used. In other embodiments, the expression vectors are used in gene therapy. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are known.

Regulatory Elements

The terms "expression construct" or "expression vector" are meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid coding sequence is capable of being transcribed. In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent and under

the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter, and glyceraldehyde-3-phosphate dehydrogenase promoter can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Where a cDNA insert is employed, typically one will typically include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed, such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression construct is a terminator. These elements can serve to enhance message levels and to minimize read through from the construct into other sequences.

Selectable Markers

In certain embodiments of the invention, the cells containing nucleic acid constructs of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants. For example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin, and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid

encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

Delivery of Expression Vectors

There are a number of ways in which expression vectors may be introduced into cells. In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome, and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). Preferred gene therapy vectors are generally viral vectors.

Although some viruses that can accept foreign genetic material are limited in the number of nucleotides they can accommodate and in the range of cells they infect, these viruses have been demonstrated to successfully effect gene expression. However, adenoviruses do not integrate their genetic material into the host genome and therefore do not require host replication for gene expression making them ideally suited for rapid, efficient, heterologous gene expression. Techniques for preparing replication infective viruses are well known in the art.

In using viral delivery systems, one will desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, animal or individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

DNA viruses used as gene vectors include the papovaviruses (e.g., simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986).

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include, but is not limited to, constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense or a sense polynucleotide that has been cloned therein.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retroviral infection, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late

promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNAs for translation.

In currently used systems, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of adenovirus vectors which are replication deficient depend on a unique helper cell line, designated 293, which is transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3, or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*,

Vero cells or other monkey embryonic mesenchymal or epithelial cells. As discussed, the preferred helper cell line is 293.

Racher *et al.*, (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) are employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking is initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking is commenced for another 72 hr.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

A typical vector applicable to practicing the present invention is replication defective and will not have an adenovirus E1 region. Thus, it is most convenient to introduce the polynucleotide encoding the gene at the position from which the E1-coding sequences have been removed. However, the position of insertion of the

construct within the adenovirus sequences is not critical. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.*, (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, e.g., 10^9 - 10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1991). Animal studies have suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

Other gene transfer vectors may be constructed from retroviruses. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, *gag*, *pol*, and *env*, that code

for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences, and also are required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding protein of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol*, and *env* genes, but without the LTR and packaging components, is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are capable of infecting a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

There are certain limitations to the use of retrovirus vectors. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This may result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the *gag*, *pol*, *env* sequence integrated in the host cell genome. However, new packaging cell lines are now

available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

Other viral vectors may be employed as expression constructs. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988), adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984), and herpes viruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990), DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection, DNA-loaded liposomes and lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers. Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo,

HeLa, and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

A number of selection systems may be used including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk-*, *hgpri-* or *aprt-* cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*: that confers resistance to methotrexate; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

Pharmaceutical compositions

Where clinical applications are contemplated, it may be necessary to prepare pharmaceutical compositions - expression vectors, virus stocks, proteins, antibodies and drugs - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of impurities that could be harmful to humans or animals.

One generally will desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also are employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the protein or peptide, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the proteins or peptides of the present

invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention are via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal, intraarterial or intravenous injection. Such compositions normally would be administered as pharmaceutically acceptable compositions, described *supra*.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various other ingredients

enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Therapeutic agents

In certain embodiments, chemotherapeutic agents may be attached to a targeting peptide or fusion protein for selective delivery to a tumor. Agents or factors suitable for use may include any chemical compound that induces DNA damage when applied to a cell. Chemotherapeutic agents include, but are not limited to, 5-fluorouracil, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin (CDDP), cyclophosphamide, dactinomycin, daunorubicin, doxorubicin, estrogen receptor binding agents, etoposide (VP16), farnesyl-protein transferase inhibitors, gemcitabine, ifosfamide, mechlorethamine, melphalan, mitomycin, navelbine, nitrosurea, plicomycin, procarbazine, raloxifene, tamoxifen, taxol, temazolomide (an aqueous form of DTIC), transplatin, vinblastine and methotrexate, vincristine, or any analog or derivative variant of the foregoing. Most chemotherapeutic agents fall into the following categories: alkylating agents, antimetabolites, antitumor antibiotics, corticosteroid hormones, mitotic inhibitors, and nitrosoureas, hormone agents, miscellaneous agents, and any analog or derivative variant thereof.

Chemotherapeutic agents and methods of administration, dosages, etc. are well known to those of skill in the art (see for example, the "Physicians Desk Reference", Goodman & Gilman's "The Pharmacological Basis of Therapeutics" and in "Remington's Pharmaceutical Sciences", incorporated herein by reference in relevant parts), and may be combined with the invention in light of the disclosures herein. Some variation in dosage will necessarily occur depending on the condition of the subject

being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Examples of specific chemotherapeutic agents and dose regimes are also described herein. Of course, all of these dosages and agents described herein are exemplary rather than limiting, and other doses or agents may be used by a skilled artisan for a specific patient or application. Any dosage in-between these points, or range derivable therein is also expected to be of use in the invention.

Alkylating agents

Alkylating agents are drugs that directly interact with genomic DNA to prevent the cancer cell from proliferating. This category of chemotherapeutic drugs represents agents that affect all phases of the cell cycle, that is, they are not phase-specific. An alkylating agent, may include, but is not limited to, a nitrogen mustard, an ethylnimenc, a methylmelamine, an alkyl sulfonate, a nitrosourea or a triazines. They include but are not limited to: busulfan, chlorambucil, cisplatin, cyclophosphamide (cytoxan), dacarbazine, ifosfamide, mechlorethamine (mustargen), and melphalan.

Antimetabolites

Antimetabolites disrupt DNA and RNA synthesis. Unlike alkylating agents, they specifically influence the cell cycle during S phase. Antimetabolites can be differentiated into various categories, such as folic acid analogs, pyrimidine analogs and purine analogs and related inhibitory compounds. Antimetabolites include but are not limited to, 5-fluorouracil (5-FU), cytarabine (Ara-C), fludarabine, gemcitabine, and methotrexate.

Natural Products

Natural products generally refer to compounds originally isolated from a natural source, and identified as having a pharmacological activity. Such compounds, analogs and derivatives thereof may be, isolated from a natural source, chemically synthesized or recombinantly produced by any technique known to those of skill in the art. Natural

products include such categories as mitotic inhibitors, antitumor antibiotics, enzymes and biological response modifiers.

Mitotic inhibitors include plant alkaloids and other natural agents that can inhibit either protein synthesis required for cell division or mitosis. They operate during a specific phase during the cell cycle. Mitotic inhibitors include, for example, docetaxel, etoposide (VP16), teniposide, paclitaxel, taxol, vinblastine, vincristine, and vinorelbine.

Taxoids are a class of related compounds isolated from the bark of the ash tree, *Taxus brevifolia*. Taxoids include but are not limited to compounds such as docetaxel and paclitaxel. Paclitaxel binds to tubulin (at a site distinct from that used by the vinca alkaloids) and promotes the assembly of microtubules.

Vinca alkaloids are a type of plant alkaloid identified to have pharmaceutical activity. They include such compounds as vinblastine (VLB) and vincristine.

Antitumor Antibiotics

Antitumor antibiotics have both antimicrobial and cytotoxic activity. These drugs also interfere with DNA by chemically inhibiting enzymes and mitosis or altering cellular membranes. These agents are not phase specific so they work in all phases of the cell cycle. Examples of antitumor antibiotics include, but are not limited to, bleomycin, dactinomycin, daunorubicin, doxorubicin (Adriamycin), plicamycin (mithramycin) and idarubicin.

Hormones

Corticosteroid hormones are considered chemotherapy drugs when they are implemented to kill or slow the growth of cancer cells. Corticosteroid hormones can increase the effectiveness of other chemotherapy agents, and consequently, they are frequently used in combination treatments. Prednisone and dexamethasone are examples of corticosteroid hormones.

Progestins such as hydroxyprogesterone caproate, medroxyprogesterone acetate, and megestrol acetate have been used in cancers of the endometrium and breast. Estrogens such as diethylstilbestrol and ethinyl estradiol have been used in cancers such as breast and prostate. Antiestrogens such as tamoxifen have been used in cancers such as breast. Androgens such as testosterone propionate and fluoxymesterone have also been used in treating breast cancer. Antiandrogens such as flutamide have been used in the treatment of prostate cancer. Gonadotropin-releasing hormone analogs such as leuprolide have been used in treating prostate cancer.

Miscellaneous Agents

Some chemotherapy agents do not fall into the previous categories based on their activities. They include, but are not limited to, platinum coordination complexes, anthracenedione, substituted urea, methyl hydrazine derivative, adrenalcortical suppressant, amsacrine, L-asparaginase, and tretinoin. It is contemplated that they may be used within the compositions and methods of the present invention.

Platinum coordination complexes include such compounds as carboplatin and cisplatin (*cis*-DDP).

An anthracenedione such as mitoxantrone has been used for treating acute granulocytic leukemia and breast cancer. A substituted urea such as hydroxyurea has been used in treating chronic granulocytic leukemia, polycythemia vera, essential thrombocytosis and malignant melanoma. A methyl hydrazine derivative such as procarbazine (N-methylhydrazine, MIH) has been used in the treatment of Hodgkin's disease. An adrenocortical suppressant such as mitotane has been used to treat adrenal cortex cancer, while aminoglutethimide has been used to treat Hodgkin's disease.

Regulators of Programmed Cell Death

Apoptosis, or programmed cell death, is an essential process for normal embryonic development; maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr et al., 1972). The Bcl-2 family of proteins and ICE-like proteases

have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Bakhshi et al., 1985; Cleary and Sklar, 1985; Cleary et al., 1986; Tsujimoto et al., 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins, which can be categorized as death agonists or death antagonists.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (e.g., Bcl_{XL}, Bcl_w, Bcl_s, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (e.g., Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

Non-limiting examples of pro-apoptosis agents contemplated within the scope of the present invention include gramicidin, magainin, mellitin, defensin, cecropin, (KLAKLAK)₂ (SEQ ID NO:1), (KLAKKLA)₂ (SEQ ID NO:2), (KAAKKAA)₂ (SEQ ID NO:3) or (KLGGKLG)₃ (SEQ ID NO:4).

Angiogenic inhibitors

In certain embodiments the present invention may concern administration of targeting peptides attached to anti-angiogenic agents, such as angiotensin, laminin peptides, fibronectin peptides, plasminogen activator inhibitors, tissue metalloproteinase inhibitors, interferons, interleukin 12, platelet factor 4, IP-10, Gro- β , thrombospondin, 2-methoxyoestradiol, proliferin-related protein, carboxamidotriazole, CM101, Marimastat, pentosan polysulphate, angiopoietin 2 (Regeneron), interferon-alpha, herbimycin A, PNU145156E, 16K prolactin fragment, Linomide, thalidomide, pentoxifylline, genistein, TNP-470, endostatin, paclitaxel, Docetaxel (Taxotere), polyamines, a proteasome inhibitor, a kinase inhibitor, a signaling inhibitor (e.g.,

SU5416 or SU6668 from Sugen, South San Francisco, CA), accutin, angiostatin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 or minocycline.

Dosages

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, and in particular to pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by the FDA Office of Biologics standards.

EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1. Biopanning with Phage Display Libraries Using Human Patients

Certain of the methods and compositions of the present invention concern identification of targeting peptides for human organs, tissues or cell types by *in vivo* biopanning. Generally, protocols used in animal subjects, such as mice, are not suited for humans. Further, ethical considerations play a large role in human protocols. The following novel methods are preferred for use with humans, although the skilled artisan

will realize that variations on the methods and compositions disclosed herein may be used within the scope of the present invention.

Human preparation

Patients were selected for the protocol according to inclusion and exclusion criteria. Inclusion criteria include: (1) patient legally declared brain dead or terminal wean patient; (2) approval of attending and/or treating physicians; and (3) approved written informed consent form signed by the patient's legally responsible family member. Exclusion criteria were: (1) the absence of a responsible family member; (2) HIV positive patient; (3) patient with active tuberculosis infection; (4) acute or chronic hepatitis B or C infections; or (5) patient was a potential organ transplant donor. In preferred embodiments, the patient was not on antibiotics for at least the previous 6 hrs, preferably the last 24 hrs, in order to avoid detrimental effects on the bacterial hosts used to propagate the phage used for the peptide display library.

After informed consent and before the patient was prepared for the procedure, relatives of the patient were asked to leave the room the patient was in. The patient had a well running IV line (preferably central) with nothing but saline running through the channel of application of the phage library. Personnel required for the procedure were notified (i.e., intervention radiologist, internist, surgeon, nurse, possibly neurologist or neuroradiologist). Materials needed for biopsies were collected: bone marrow aspiration needle, lumbar puncture kit, skin biopsy kit, materials for taking biopsies of any organ, tissue or cell type used for targeted peptide identification, such as liver, fat and tumor, materials for transabdominal prostate biopsy, 50 ml syringe with 40 ml saline for blood sample, 10 ml tube containing heparin and 10 ml serum collection tube to draw blood sample for lab tests. Before phage library injection, blood samples were drawn for routine screening of liver function, bicarbonate, electrolytes and blood count, unless test results from the day of the injection were available.

In the laboratory, 120 large dishes with LB-tet/kan agar as well as 200 regular LB tet/kan plates (100 mm) were prepared (tetracycline concentration = 40 $\mu\text{g/ml}$, kanamycin concentration = 50 $\mu\text{g/ml}$). *E. coli* K91 kan were grown in 10 independent

tubes, each containing 10 ml TB medium plus supplements. Growth of bacteria was started approx. 15-60 min prior to beginning the biopsies. About 10^{14} TU of the (preferably primary) phage library were diluted in 200 ml ringer lactate at room temperature and aspirated under clean but not necessarily sterile conditions into four 50 ml syringes. LB-tet/kan dishes or plates were warmed in a 37°C incubator. One liter of LB medium containing 0.2 µg/ml tet and 100 µg/ml kan was warmed in the waterbath at 37°C. One liter LB medium containing 40 µg/ml tet and 100 µg/ml kan was warmed to 37°C and 8 more liters were prepared at room temperature. Thirty glass grinders A and B size as well as suitable glass tubes were autoclaved. Three 50 ml Falcon tubes were prepared for each of the organs for which biopsies were to be taken. Tubes were filled with 10 ml DMEM-PI - DMEM containing PMSF (1 mM), aprotinin (20µg/ml) and leupeptin (1µg/ml) - and put on ice approximately 15 minutes before tissue collection. For each of the 4 teams taking over in the lab after the tissue samples were collected, one autoclaved set of surgicals (i.e., at least one forceps and one pair of scissors and a scalpel) were prepared in order to trim, divide or mince organ samples.

Phage library injection

All drugs running through the intended port of application of the phage library were discontinued during library injection. If possible without compromising the patient's hemodynamic stability, all IV drugs running through different ports were discontinued during library injection as well. A running saline infusion ensured that the IV line for the library injection was open and was left running during the injection.

The 200 ml library solution was manually injected over a period of 10 minutes while monitoring and protocoling the patient's vital functions such as breathing (if not mechanically ventilated), heart rate and blood pressure. The injection was stopped any time the running saline infusion stopped dripping, indicating obstruction of the line. Fifteen minutes after beginning the injection, tissue sample collection (biopsies) was initiated. Preferred biopsy sites included bone marrow aspirate, liver, prostate, skin, skeletal muscle, tumor (if applicable), adipose tissue, blood (as positive control), blood (for red/white blood cells) and cerebral-spinal fluid (CSF).

The samples were taken under very clean if not sterile conditions to reduce contamination with bacteria. To the extent possible, the different samples were taken simultaneously. For small samples, triplicate biopsies were preferred. The time elapsed between beginning of injection and the collection of a particular tissue sample was recorded. Tissue samples were placed in the prepared 50 ml tubes containing DMEM-PI and stored on ice. For bone marrow, a regular diagnostic sample (undiluted into a syringe with heparin) was taken in addition to the samples diluted in 40 ml saline to confirm aspiration of bone marrow as opposed to blood. If needed, all IV drugs, including antibiotics, were continued after removal of tissue samples.

All organ samples that were not taken in triplicate were divided under clean conditions to obtain three different pieces of tissue. The three samples of each organ were handled as follows. One piece was stored at -80°C as a backup. One piece was forwarded to the histology/pathology department to cut cryosections (or to make smears for bone marrow) and perform HE staining (Pappenheim staining for bone marrow) as well as phage staining to confirm that the samples contained the organ of interest. In some cases the histology sample was divided in two - one for regular HE staining and one for LCM (laser capture microscopy) or LPC (laser pressure catapulting). The last of the three original pieces was processed for bacterial infection to recover phage.

After freezing of backup tissue and saving material for pathology, samples for phage rescue were weighed. Samples were kept on ice at all times. Sample was transferred to 1ml DMEM-PI in a glass tube and homogenized with a grinder. Some organs such as bone marrow, blood, or CSF do not require homogenization, whereas other organs like muscle need to be minced before they can be efficiently homogenized. Lysis of erythrocytes for blood samples was preferred. Homogenized samples were transferred to autoclaved 2 ml Eppendorf tubes.

Tissue samples were washed 3 times with ice cold DMEM-PI containing 1% BSA by mixing the tissue with DMEM-PI and vortexing for 30 seconds. After centrifugation at 4,000 rpm for 3 min, supernatant was carefully discarded, leaving the tissue pellet undisturbed. A small amount of medium was left on the surface of the

pellet. Samples were vortexed again for 30 seconds before adding more medium to facilitate resuspension of the tissue. After adding 1.5 ml of DMEM-PI plus BSA the samples were centrifuged again. When processing multiple samples, the tissues were kept on ice at all times.

After 3 washes, the pellet was briefly vortexed and the dissolved pellet was warmed *briefly* to 37°C before adding bacteria. The washed tissue samples were incubated with 1.5 ml of competent K91-kan bacteria (OD₆₀₀ 0.2 in 1:10 dil.) for one hour at room temperature, then transferred to Falcon tubes containing 10 ml of LB medium with 0.2 µg/ml tetracycline. After 20 min at RT, multiple aliquots were plated on LB tet/kan plates or dishes containing 40 µg/ml of tetracycline and 100 µg/ml kanamycin. The following quantities (per organ sample) were plated: 2 dishes with 3 ml; 2 dishes with 1 ml; 3 dishes with 300 µl; 3 dishes with 100 µl; 3 dishes with 30 µl.

The beads that were used for plating were passed on to two subsequent 10 cm LB tet/kan plates to recover every potentially phage infected bacterial clone that might be trapped on the bead surface. Dishes were incubated overnight at 37°C.

The remaining 2-3 ml of infected culture (including the homogenized tissue) was transferred to 10 ml of LB medium containing 40 µg/ml tetracycline and 100 µg/ml kanamycin (LB tet/kan) and shaken at 37°C for 2 hr. This approximately 12 ml culture was transferred to 1 liter LB tet/kan and grown overnight in a 37°C shaker.

The next day, phage were rescued from the bulk amplified bacterial culture according to standard protocols and saved for a potential second round of *in vivo* selection. From the plates/dishes in the incubator, 1500 well separated colonies were picked for each organ plated and transferred to 96 well plates containing 20 µl PBS/well for sequencing. This assumed a readout of about 2 out of 3 picked colonies to obtain 1000 sequences.

After picking 1500 colonies, the remainder of colonies on the dishes/plates were grown in 1000 ml LB tet/kan overnight in the 37°C shaker. Then phage were harvested as before for a second round of selection. Alternatively, the plates were stored in the refrigerator and 1000-2000 individual colonies grown at a time. Alternatively, the

remainder of colonies were transferred to PBS and stored frozen to infect and amplify as needed.

Numerous non-limiting examples of human organ, tissue or cell type selective targeting peptides have been identified by *in vivo* biopanning using the present methods, as disclosed below. A non-limiting example of human biopanning using the methods of the present invention, along with targeting peptide sequences identified by the present methods, is disclosed in Example 3.

Example 2. Mapping the Human Vasculature by *In vivo* Phage Display

The *in vivo* selection method was used to screen a phage library in a human subject. A pattern recognition analysis program was used to survey 47,160 tripeptide motifs within peptides that localized to the human bone marrow, fat, skeletal muscle, prostate, or skin. The results of this large-scale screening indicated that the distribution of circulating peptide motifs to different organs is non-random. High-throughput analysis of peptide motifs enriched in individual tissues revealed similarities to sequences present in candidate ligands for differentially expressed vascular receptors.

These data represent a major step towards the construction of a ligand-receptor map of human vasculature and may have broad implications for the development of targeted therapies. Many therapeutic targets may be expressed in very restricted--but highly specific and accessible--locations in the vascular endothelium. Potential targets for intervention may be overlooked in high-throughput DNA sequencing or in gene arrays because these approaches do not usually take into account cellular location and anatomical, and functional context. The human *in vivo* phage display screening methods disclosed herein are uniquely suited to identification of naturally occurring ligand-receptor pairs that may provide the basis for highly selective therapies against various disease states.

Materials and Methods

A 48 year-old male Caucasian patient who had been diagnosed with Waldenström macroglobulinemia (a B cell malignancy) was previously treated by splenectomy, systemic chemotherapy (fludarabine, mitoxantrone, and dexamethasone), and immunotherapy (anti-CD20 monoclonal antibody). In the few months prior to his admission, the disease became refractory to treatment and clinical progression occurred with retroperitoneal lymphadenopathy, pancytopenia, and marked bone marrow infiltration by tumor cells. The patient was admitted with massive intracranial bleeding secondary to thrombocytopenia. Despite prompt craniotomy and surgical evacuation of a cerebral hematoma, the patient remained comatose with progressive and irreversible loss of brainstem function until the patient met the formal criteria for brain-based determination of death, as evaluated by an independent clinical neurologist. Because of his advanced cancer, the patient was rejected as transplant organ donor. After surrogate written informed consent was obtained from the legal next of kin, *in vivo* phage display was performed.

In Vivo Phage Display

A large-scale preparation of a CX₇C (C, cysteine; X, any amino acid residue) phage display random peptide library was optimized to create the highest possible insert diversity (Pasqualini *et al.*, 2000). The diversity of the library was about 2×10^8 and its final titer was about 10^{12} transducing units (TU)/ml. Short-term intravenous infusion of the phage library (a total dose of 10^{14} phage TU suspended in 100 ml of saline) into the patient was followed by multiple representative tissue biopsies. Prostate and liver samples were obtained by needle biopsy under ultrasonographic guidance. Skin, fat tissue, and skeletal muscle samples were obtained by surgical excision. Bone marrow needle aspirates and core biopsies were also obtained. Histopathological diagnosis was determined by examination of frozen sections processed from tissues obtained at the bedside.

Triplicate samples were processed for host bacterial infection, phage recovery, and histopathological analysis. In brief, tissues were weighed, ground with a glass

Dounce homogenizer, suspended in 1 ml of Dulbecco Modified Eagle's medium (DMEM) containing proteinase inhibitors (DMEM-prin; 1 mM PMSF, 20 μ g/ml aprotinin, and 1 μ g/ml leupeptin), vortexed, and washed three times with DMEM-prin. The human tissue homogenates were incubated with 1 ml of host bacteria (log phase *E. coli* K91kan; OD₆₀₀ ~ 2). Aliquots of the bacterial culture were plated onto Luria-Bertani agar plates containing 40 μ g/ml tetracycline and 100 μ g/ml of kanamycin. Plates were incubated overnight at 37°C. Bacterial colonies were processed for sequencing of phage inserts recovered from each tissue and from unselected phage library. Human samples were handled with universal blood and body fluid precautions.

Statistical Analysis

A high-throughput character pattern recognition program (M.D. Anderson Cancer Center, Biostatistics, Houston, TX) was developed to automate the analysis of the peptide motifs derived from phage screenings. By using SAS (version 8, SAS Institute) and Perl (version 5.0), the program conducts an exhaustive amino acid residue sequence count and tracks the relative frequencies of N distinct tripeptide motifs representing all possible n_3 overlapping tripeptide motifs in both directions ($N \ll n_3$). This was applied for phage recovered from each target tissue and for the unselected CX₇C random phage display peptide library.

With "p" defined as the probability of observing a particular tripeptide motif under total randomness, and $q=1-p$, the probability of observing K sequences characterized as a particular tripeptide motif out of n_3 total tripeptide motif sequences is binomial (n_3, p). That probability may be approximated by the formula: $p_K = \Phi[(k+1)/\sqrt{n_3 pq}] - \Phi[k/\sqrt{n_3 pq}]$, where Φ is the cumulative Gaussian probability. The value p_K may be treated as a P -value in testing for total randomness of observing exactly K sequences of a particular tripeptide motif. However, this test requires exact knowledge of the true value of p , which it is difficult to obtain in practice.

In order to identify the motifs that were enriched in the screening, the count for each tripeptide motif within each tissue was compared with the count for that tripeptide

motif within the unselected library. Starting from a CX₇C peptide insert, counts were recorded for all overlapping interior tripeptide motifs, subject only to reflection and single-voting restrictions. No peptide was allowed to contribute more than once for a single tripeptide motif (or a reflected tripeptide motif). Each peptide contributed five tripeptide motifs. Tripeptide motif counts were conditioned on the total number for all motifs being held fixed within a tissue. The significance of association of a given allocation of counts was assessed by Fisher's exact test (one-tailed). Results were considered statistically significant at $P < 0.05$. In summary, to test for randomness of distribution, the relative frequencies of a particular tripeptide motif from each target was compared to the frequencies of the motifs from the unselected library. This approach is a large-scale contingency table association test.

Results

The general procedure followed in human phage display biopanning is illustrated in FIG. 1A. For biopanning with human subjects, use of a large-scale phage display library (diversity about 2×10^8) is advantageous compared to the smaller scale libraries used in mouse studies. The protocol followed was as described above.

The feasibility of producing phage display random peptide libraries in very large-scale and of selecting phage clones that home to different human organs *in vivo* through the systemic circulation is shown in FIG. 1B. Phage localizing to fat, skin, bone marrow, skeletal muscle, prostate and liver were recovered from a human subject (see FIG. 2). This is the first demonstration of the feasibility of *in vivo* phage display in humans.

To determine the distribution of the peptide inserts homing to specific sites after intravenous administration, the relative frequencies of every tripeptide motif from each target tissue were compared to the frequencies from the unselected library. The 4,716 phage inserts recovered from representative samples of five tissues (bone marrow, fat, skeletal muscle, prostate, and skin) and from the unselected library were analyzed. Tripeptide motifs were chosen for the phage insert analysis because three amino acid residues appear to provide the minimal framework for structural formation and protein-

protein interaction (Vendruscolo *et al.*, 2001). Examples of biochemical recognition units and binding of tripeptide ligand motifs to receptors include RGD (Ruoslahti, 1996), LDV (Ruoslahti, 1996), and LLG (Koivunen *et al.*, 2001) to integrins, NGR (Pasqualini *et al.*, 2000) to aminopeptidase N/CD13, and GFE (Rajotte and Ruoslahti, 1999) to membrane dipeptidase.

Each phage insert analyzed contained seven amino acid residues and contributed five potential tripeptide motifs. Taking reflection into account, a total of 47,160 tripeptide motifs were surveyed. Comparisons of the motif frequencies in a given organ relative to those frequencies in the unselected library showed the non-random nature of the peptide distribution (Table 3). This is particularly noteworthy given that only a single round of *in vivo* screening was performed. Of the tripeptide motifs enriched in tissues, some were enriched in a single site

Table 3. Peptide motifs isolated by *in vivo* phage display in humans

Target organ/motif	Motif frequency	P-value
Unselected Library		
none	N/A	N/A
<u>Bone Marrow</u>		
GFS*	1.1	0.0244
LWS*	1.0	0.0453
ARL	1.0	0.0453
FGG	1.0	0.0453
GVL	1.1	0.0453
SGT	2.3	0.0137
GGG*	2.3	0.0350
<u>Fat</u>		
EGG*	1.3	0.0400
LLV*	1.0	0.0269
LSP*	0.9	0.0402
EGR	1.1	0.0180
FGV	0.9	0.0402
<u>Muscle</u>		
LVS*	2.1	0.0036
GER	0.9	0.0344
<u>Prostate</u>		
AGG*	2.5	0.0340
EGR	1.0	0.0185
GER	0.9	0.0382
GVL	2.3	0.0079
<u>Skin</u>		
GRR*	2.9	0.0047
GGH*	0.9	0.0341
GTV*	0.8	0.0497
ARL	0.8	0.0497
FGG	1.3	0.0076
FGV	1.0	0.0234
SGT	1.0	0.0234

whereas others were enriched in multiple sites. The data are consistent with some of the peptides homing in a tissue-specific manner while other peptides targeted several tissues.

Table 3 lists motifs occurring in peptides isolated from target organs, but not from the unselected phage library (Fisher's exact test, one-tailed; $P < 0.05$). The number of peptide sequences analyzed per organ was: unselected library, 446; bone marrow, 521; fat, 901; muscle, 850; prostate, 1018; skin, 980. Asterisks indicate motifs that were enriched only in a single tissue. The abbreviation N/A means not applicable.

Tripeptide motifs appeared to be targeted to specific human organs, tissues or cell types including bone marrow (GGG, SEQ ID NO:5; GFS, SEQ ID NO:6; LWS, SEQ ID NO:130), adipose tissue (EGG, SEQ ID NO:7; LSP, SEQ ID NO:8; LLV, SEQ ID NO:9), skeletal muscle (LVS, SEQ ID NO:10), prostate (AGG, SEQ ID NO:11) and skin (GRR, SEQ ID NO:12; GGH, SEQ ID NO:13; GTV, SEQ ID NO:14) (FIG. 2A, Table 3). Tripeptide motifs that appeared to be targeted to multiple organs, tissues or cell types included GVL (SEQ ID NO:15), EGR (SEQ ID NO:16), FGV (SEQ ID NO:17), FGG (SEQ ID NO:18), GER (SEQ ID NO:19) SGT (SEQ ID NO:20) and ARL (SEQ ID NO:21) (FIG. 2B, Table 3). Each of these motifs showed a statistically significant localization to one or more target organs, tissues or cell types, compared to the unselected phage display library.

The ClustalW program (European Molecular Biology Laboratory; EMBL) was used to analyze the original cyclic phage peptide inserts of seven amino acid residues containing the tripeptide motifs. The analysis revealed four to six residue motifs that were shared among multiple peptides isolated from a given organ (FIG. 2A, Table 4). Each of the motifs was searched for in on-line databases (including BLAST, SWISSPROT, PROSITE, PRODOM, and BLOCKS) through the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html#protein_databases). Some appeared within previously known human proteins and others were not found in the databases. As these motifs are likely to represent sequences present in circulating

ligands (either secreted proteins or surface receptors in circulating cells) that home to vascular receptors, a panel of candidate human proteins potentially mimicked by selected peptide motifs was compiled (Table 4).

Table 4 Examples of human proteins potentially mimicked by peptide motifs

Extended motif	Human protein	Protein description	Accession number
<u>Bone</u>			
<u>Marrow</u>			
PGGG	Bone morphogenetic protein 3B	growth, factor, TGF-beta family member	NP_004953
PGGG	Fibulin 3	fibrillin- and EGF-like	Q12805
GHHSFG	Microsialin	macrophage antigen, glycoprotein	NP_001242
<u>Fat</u>			
EGGT	LTBP-2	fibrillin- and EGF-like, TGF-beta Interactor	CAA86030
TGGE	Sortilin	adipocyte differentiation-induced receptor	CAA66904
GPSLH	Protocadherin gamma C3	cell adhesion	AAD43784
<u>Muscle</u>			
GGSVL	ICAM-1	intercellular adhesion molecule	P05362
LVSGY	Ft4	endothelial growth factor receptor	CAA48290
<u>Prostate</u>			
RRAGGS	Interleukin 11	cytokine	NP_000632
RRAGG	Smad6	Smad family member	AAB94137
<u>Skin</u>			
GRRG	TGF-beta 1	growth factor, TGF-beta family member	XP_008912
HGG+G	Neuropilin-1	endothelial growth factor receptor	AAF44344
+PHGG	Pentaxin	infection/trauma-induced glycoprotein	CAA45158
PHGG	Macrophage inhibitory cytokine-1	growth, factor, TGF-beta family member	AAB88673
+PHGG	Desmoglein 2	epithelial cell junction protein	S38673
VTG+SG	Desmoglein 1	epidermal cell junction protein	AAC83817

<u>Multiple</u>			
<u>Organs</u>			
EGRG	MMP-9	gelatinase	AAH06093
GRGE	ESM-1	endothelial cell-specific molecule	XP_003781
NFGVV	CDO	surface glycoprotein, Ig- and fibronectin-like	NP_058648
GERIS	BPA1	basement membrane protein	NP_001714
SIREG	Wnt-16	glycoprotein	Q9UBV4
+GVLW	Sialoadhesin	Ig-like lectin	AAK00757
WLVG+	IL-5 receptor	soluble interleukin 5 receptor	CAA44081
GGFR	Plectin 1	endothelial focal junction-localized protein	CA91196
GGFF	TRANCE	TNF family member	AAC51762
+SGGF	MEGF8	EGF-like protein	T00209
PSGTS	ICAM-4	intercellular adhesion glycoprotein	Q14773
+TGSP	Perlecan	vascular repair heparan sulfate proteoglycan	XP_001825

Extended targeting motifs homologous to known proteins were identified from bone marrow (GHHSFG, SEQ ID NO:22; PGGG, SEQ ID NO:23), fat (EGGT, SEQ ID NO:24; TGGE, SEQ ID NO:25; GPSLH, SEQ ID NO:26), skeletal muscle (GGSVL, SEQ ID NO:27; LVSGY, SEQ ID NO:28), prostate (RRAGGS, SEQ ID NO:29; RRAGG, SEQ ID NO:30), skin (GRRG, SEQ ID NO:31; HGGXG, SEQ ID NO:32; PHGG, SEQ ID NO:33; VTGXSG, SEQ ID NO:34) and from multiple organs (GRGE, SEQ ID NO:35; NFGVV, SEQ ID NO:36; GERIS, SEQ ID NO:37; SIREG, SEQ ID

NO:38; GVLW, SEQ ID NO:39; WLVG, SEQ ID NO:40; GGFR, SEQ ID NO:41; GGFF, SEQ ID NO:42; SGGF, SEQ ID NO:43; PSGTS, SEQ ID NO:44; TGSP, SEQ ID NO:45, EGRG, SEQ ID NO:131).

Table 4 shows sequences corresponding to regions of 100% sequence identity between the peptide selected and the candidate protein. Conserved amino acid substitutions are indicated as by a "+". Tripeptide motifs from Table 3 are shown in bold.

The homologous proteins thus identified may represent natural ligands for the human receptors that bound targeting phage. For example, a peptide contained within bone morphogenetic protein 3B (BMP 3B) was recovered from phage localized to bone marrow. BMP 3 B is a growth factor that is known to regulate bone development (Daluiski *et al.*, 2001). Thus, this protein is a candidate ligand mimicked by a peptide homing to bone marrow tissue. Also, interleukin 11 has been shown to interact with receptors within endothelium and prostate epithelium (Mahboubi *et al.*, 2000). IL-11 may be mimicked by a targeting peptide recovered from the prostate (Table 4). In addition to secreted ligands, motifs were also found in several extracellular or transmembrane proteins that may operate selectively in the target tissue, such as sortilin in fat (Lin *et al.*, 1997). Certain motifs appear to be enriched in multiple organs. One such peptide is a candidate mimotope of perlecan (Table 4), which is a protein known to maintain vascular homeostasis.

These results were confirmed by *in situ* staining, using polyclonal antibodies against IL-11 receptor alpha. IL-11 is a cytokine that is apparently mimicked by the peptide motif RRAGGS (SEQ ID NO:29), a human prostate targeting peptide. This suggests that the IL-11 alpha receptor (IL-11R α) should be overexpressed in prostate blood vessels. Studies with cultured cells have shown that IL-11 interacts with receptors in endothelium and prostate epithelium (Mahboubi *et al.*, 2000; Campbell *et al.*, 2001). However, expression of IL-11R α in prostate blood vessels has not previously been examined.

Immunostaining of prostate thin sections with antibodies against IL-11R α showed that IL-11R α is present in the luminal prostate epithelium and in prostate blood vessels (not shown). This result validates the human biopanning results and shows that the presence of cell surface receptors identified by targeting peptide binding can be confirmed by antibodies against the receptor protein.

In vivo phage display in humans may reveal heterogeneity at the level of individual patients. For example, it is unclear whether the malignant B cell infiltration in the tested patient influenced the outcome of the screening of the bone marrow, given that the patient had Waldenström macroglobulinemia. However, if vascular targeting ligands are to be developed for clinical use, they must resist degradation and be robust enough to target blood vessels in diseased tissue *in vivo*. Thus, a considerable advantage of the method described here is that the selected targeting peptides bind to native receptors, as they are expressed *in vivo*. Even if a ligand-receptor interaction is mediated through a conformational (rather than a linear) epitope, it is still possible to select binders in the screening. As it is difficult to ensure that transmembrane proteins expressed by recombinant systems (such as in protein arrays) maintain the correct structure and folding after purification *in vitro*, peptides selected *in vivo* is likely to be more suitable to clinical applications, such as identification of novel inhibitors or activators of native receptor proteins.

Precedent exists to suggest that phage can be safely administered to patients, as bacteriophage were widely used in humans during the pre-antibiotic era. Ultimately, it may be possible to determine molecular profiles of human blood vessels by infusing phage libraries systemically before resections of lung, prostate, breast, or colorectal carcinomas or even regionally before resection of limb sarcomas. The methods disclosed herein will allow the construction of a molecular map outlining vascular diversity in each human organ, tissue, or disease.

Human targeting peptide sequences identified by the methods described above and directed to fat, skeletal muscle, skin bone marrow, prostate or to multiple organs are listed in Table 5.

Table 5. Human Targeting Peptide Sequences

Sequence	SEQ ID	Tissue
AEEGGS	SEQ ID NO:46	Fat
EGGSFNW	SEQ ID NO:47	Fat
IEGGQVG	SEQ ID NO:48	Fat
EGGSVES	SEQ ID NO:49	Fat
EGGIFWH	SEQ ID NO:50	Fat
EGGLSGC	SEQ ID NO:51	Fat
CAEGGAS	SEQ ID NO:52	Fat
AEGGVRG	SEQ ID NO:53	Fat
AEGGRVY	SEQ ID NO:54	Fat
VVEGGVK	SEQ ID NO:55	Fat
VLVGEGG	SEQ ID NO:56	Fat
TKKLEGG	SEQ ID NO:57	Fat
GGLSPNW	SEQ ID NO:58	Fat
TGHLSPG	SEQ ID NO:59	Fat
VLSPGLG	SEQ ID NO:60	Fat
LSPGVKG	SEQ ID NO:61	Fat
LSPWKRR	SEQ ID NO:62	Fat
AWLSPAR	SEQ ID NO:63	Fat
AWRRLSP	SEQ ID NO:64	Fat

LSPDDAL	SEQ ID NO:65	Fat
LVSGGMA	SEQ ID NO:66	Skeletal muscle
LVSGCNT	SEQ ID NO:67	Skeletal muscle
DLVSGYG	SEQ ID NO:68	Skeletal muscle
LVSTSAT	SEQ ID NO:69	Skeletal muscle
TALVSQT	SEQ ID NO:70	Skeletal muscle
WLVSGIG	SEQ ID NO:71	Skeletal muscle
LVSSVIP	SEQ ID NO:72	Skeletal muscle
PSLVSSV	SEQ ID NO:73	Skeletal muscle
GVSLVST	SEQ ID NO:74	Skeletal muscle
QLVSGEP	SEQ ID NO:75	Skeletal muscle
NLVSRRL	SEQ ID NO:76	Skeletal muscle
LVSWRGS	SEQ ID NO:77	Skeletal muscle
DHFLVSP	SEQ ID NO:78	Skeletal muscle
GRGLVSL	SEQ ID NO:79	Skeletal muscle
FPVALVS	SEQ ID NO:80	Skeletal muscle
RWSSLVS	SEQ ID NO:81	Skeletal muscle
WSKSLVS	SEQ ID NO:82	Skeletal muscle
PGRSLVS	SEQ ID NO:83	Skeletal muscle
GRRGSAG	SEQ ID NO:84	Skin
RPGRRGS	SEQ ID NO:85	Skin
SGRRGPR	SEQ ID NO:86	Skin
GLGRRNG	SEQ ID NO:87	Skin

GGRRSQT	SEQ ID NO:88	Skin
LWDGRRH	SEQ ID NO:89	Skin
GRRSVLT	SEQ ID NO:90	Skin
FGRRNLF	SEQ ID NO:91	Skin
GAGRRYW	SEQ ID NO:92	Skin
GRRLWAT	SEQ ID NO:93	Skin
GVGRRFG	SEQ ID NO:94	Skin
LEMVGRR	SEQ ID NO:95	Skin
LSSIGRR	SEQ ID NO:96	Skin
GRRWIDV	SEQ ID NO:97	Skin
GRREEGL	SEQ ID NO:98	Skin
GRRVLGR	SEQ ID NO:99	Skin
RGLMGRR	SEQ ID NO:100	Skin
RFLGRR	SEQ ID NO:101	Skin
PGVGRRRL	SEQ ID NO:102	Skin
GVIDGRR	SEQ ID NO:103	Skin
ADGRRLG	SEQ ID NO:104	Skin
AGRRAQI	SEQ ID NO:105	Skin
YGRRARE	SEQ ID NO:106	Skin
PGRRLRM	SEQ ID NO:107	Skin
GGRRVTL	SEQ ID NO:108	Skin
EQGGRRRL	SEQ ID NO:109	Skin
SGRRLHP	SEQ ID NO:110	Skin

FDHSGRR	SEQ ID NO:111	Skin
GRRDVAI	SEQ ID NO:112	Skin
GGHPRLA	SEQ ID NO:113	Skin
GGHWRVN	SEQ ID NO:114	Skin
GGHILEV	SEQ ID NO:115	Skin
GGHRAQS	SEQ ID NO:116	Skin
GDGGHRP	SEQ ID NO:117	Skin
SCVGGHS	SEQ ID NO:118	Skin
GSGVGGH	SEQ ID NO:119	Skin
VRGWGGH	SEQ ID NO:120	Skin
WRGWGGH	SEQ ID NO:121	Skin
WGSKGTV	SEQ ID NO:122	Skin
TGSLGTV	SEQ ID NO:123	Skin
WGTVSDA	SEQ ID NO:124	Skin
ATGTVGP	SEQ ID NO:125	Skin
VVGTVAW	SEQ ID NO:126	Skin
WVVGTVT	SEQ ID NO:127	Skin
RVVHGTV	SEQ ID NO:128	Skin
GTVRFFS	SEQ ID NO:129	Skin
SGGGPGV	SEQ ID NO:132	Bone Marrow
RLGGGLA	SEQ ID NO:133	Bone Marrow
WWGGGVS	SEQ ID NO:134	Bone Marrow
GSARGGG	SEQ ID NO:135	Bone Marrow

ARGGGIR	SEQ ID NO:136	Bone Marrow
RAAGGGG	SEQ ID NO:137	Bone Marrow
GSSAGGG	SEQ ID NO:138	Bone Marrow
LGEAGGG	SEQ ID NO:139	Bone Marrow
GGLEGGG	SEQ ID NO:140	Bone Marrow
GNGGGES	SEQ ID NO:141	Bone Marrow
STGGGCS	SEQ ID NO:142	Bone Marrow
LGGGEW	SEQ ID NO:143	Bone Marrow
HGFSHHG	SEQ ID NO:144	Bone Marrow
RRGFSLG	SEQ ID NO:145	Bone Marrow
GGFSPWL	SEQ ID NO:146	Bone Marrow
GRLVGFS	SEQ ID NO:147	Bone Marrow
TTGVGFS	SEQ ID NO:148	Bone Marrow
GRRAGGS	SEQ ID NO:149	Prostate
TRRAGGG	SEQ ID NO:150	Prostate
SRAGGLG	SEQ ID NO:151	Prostate
SYAGGLG	SEQ ID NO:152	Prostate
DVAGGLG	SEQ ID NO:153	Prostate
GAGGLGA	SEQ ID NO:154	Prostate
GAGGWGV	SEQ ID NO:155	Prostate
AGGTFKP	SEQ ID NO:156	Prostate
LGEVAGG	SEQ ID NO:157	Prostate
GSNDAGG	SEQ ID NO:158	Prostate

YRGIAGG	SEQ ID NO:159	Prostate
AGGVAGG	SEQ ID NO:160	Prostate
GGLAGGF	SEQ ID NO:161	Prostate
LLAGGVL	SEQ ID NO:162	Prostate
LVVSAGG	SEQ ID NO:163	Prostate
RTQAGGV	SEQ ID NO:164	Prostate
AGGFGEQ	SEQ ID NO:165	Prostate
AGGLIDV	SEQ ID NO:166	Prostate
AGGSTWT	SEQ ID NO:167	Prostate
AGGDWWW	SEQ ID NO:168	Prostate
AGGGLLM	SEQ ID NO:169	Prostate
VAAGGGL	SEQ ID NO:170	Prostate
LYGAGGS	SEQ ID NO:171	Prostate
CALAGGC	SEQ ID NO:172	Prostate
IGAGGVH	SEQ ID NO:173	Prostate
PKHGV LW	SEQ ID NO:174	Multiple Organ
SGVLWYH	SEQ ID NO:175	Multiple Organ
GVLWAFG	SEQ ID NO:176	Multiple Organ
QARGVLW	SEQ ID NO:177	Multiple Organ
GVLVSRM	SEQ ID NO:178	Multiple Organ
GTVGVLV	SEQ ID NO:179	Multiple Organ
VGVLPA	SEQ ID NO:180	Multiple Organ
GGVLLS	SEQ ID NO:181	Multiple Organ

SGVLIHD	SEQ ID NO:182	Multiple Organ
PYFGVLA	SEQ ID NO:183	Multiple Organ
FFVSGVL	SEQ ID NO:184	Multiple Organ
LLAGGVL	SEQ ID NO:185	Multiple Organ
GEMGGVL	SEQ ID NO:186	Multiple Organ
GRAYGVL	SEQ ID NO:187	Multiple Organ
SGVLDGR	SEQ ID NO:188	Multiple Organ
WSGGVLH	SEQ ID NO:189	Multiple Organ
WSGGVLH	SEQ ID NO:190	Multiple Organ
SRQGVLR	SEQ ID NO:191	Multiple Organ
GVLTSYQ	SEQ ID NO:192	Multiple Organ
RGVLT SQ	SEQ ID NO:193	Multiple Organ
RVPGVLS	SEQ ID NO:194	Multiple Organ
LGVLSYR	SEQ ID NO:195	Multiple Organ
KRGVLGW	SEQ ID NO:196	Multiple Organ
GVLGLGF	SEQ ID NO:197	Multiple Organ
FLGVLGR	SEQ ID NO:198	Multiple Organ
EGVLETS	SEQ ID NO:199	Multiple Organ
WWGGVLG	SEQ ID NO:200	Multiple Organ
VWSRGVL	SEQ ID NO:201	Multiple Organ
GVLRGVS	SEQ ID NO:202	Multiple Organ
SFGVLRG	SEQ ID NO:203	Multiple Organ
KGSVGVL	SEQ ID NO:204	Multiple Organ

GGHFGVL	SEQ ID NO:205	Multiple Organ
WMDVGVL	SEQ ID NO:206	Multiple Organ
AFRVGVL	SEQ ID NO:207	Multiple Organ
GVGVLRK	SEQ ID NO:208	Multiple Organ
MEGRGAG	SEQ ID NO:209	Multiple Organ
SEGRGFM	SEQ ID NO:210	Multiple Organ
VEGRNSK	SEQ ID NO:211	Multiple Organ
VEGRYTP	SEQ ID NO:212	Multiple Organ
FNBGRQM	SEQ ID NO:213	Multiple Organ
FEGRSRS	SEQ ID NO:214	Multiple Organ
DHVVGR	SEQ ID NO:215	Multiple Organ
WDGTEGR	SEQ ID NO:216	Multiple Organ
LDWREGR	SEQ ID NO:217	Multiple Organ
RGCEGRV	SEQ ID NO:218	Multiple Organ
MTPEGRV	SEQ ID NO:219	Multiple Organ
RLFEGRV	SEQ ID NO:220	Multiple Organ
REGRRMC	SEQ ID NO:221	Multiple Organ
TQFEGRR	SEQ ID NO:222	Multiple Organ
SMEGRMF	SEQ ID NO:223	Multiple Organ
PGSAEGR	SEQ ID NO:224	Multiple Organ
GEGRILA	SEQ ID NO:225	Multiple Organ
EGRFSAW	SEQ ID NO:226	Multiple Organ
EGRSDIW	SEQ ID NO:227	Multiple Organ

EGRARWL	SEQ ID NO:228	Multiple Organ
EGRERWR	SEQ ID NO:229	Multiple Organ
CQCGFGV	SEQ ID NO:230	Multiple Organ
RGGFGVR	SEQ ID NO:231	Multiple Organ
AVGFGVI	SEQ ID NO:232	Multiple Organ
AVGFGVI	SEQ ID NO:233	Multiple Organ
IVGFGVA	SEQ ID NO:234	Multiple Organ
GNFGVVW	SEQ ID NO:235	Multiple Organ
DEPFGVA	SEQ ID NO:236	Multiple Organ
VWFGVGS	SEQ ID NO:237	Multiple Organ
WFGVSL	SEQ ID NO:238	Multiple Organ
FGVGQWA	SEQ ID NO:239	Multiple Organ
SMRFGVS	SEQ ID NO:240	Multiple Organ
RFGVWTG	SEQ ID NO:241	Multiple Organ
RFGVGRV	SEQ ID NO:242	Multiple Organ
SGLFGVY	SEQ ID NO:243	Multiple Organ
MKGVFGV	SEQ ID NO:244	Multiple Organ
AFGVVSD	SEQ ID NO:245	Multiple Organ
LYAFGVV	SEQ ID NO:246	Multiple Organ
KVFGVVE	SEQ ID NO:247	Multiple Organ
FGVRTDL	SEQ ID NO:248	Multiple Organ
TIFGVRR	SEQ ID NO:249	Multiple Organ
VWPRFGG	SEQ ID NO:250	Multiple Organ

SRFGGRV	SEQ ID NO:251	Multiple Organ
MKFGGRL	SEQ ID NO:252	Multiple Organ
RFGGALR	SEQ ID NO:253	Multiple Organ
ERFGGDE	SEQ ID NO:254	Multiple Organ
FGGSVAP	SEQ ID NO:255	Multiple Organ
WFGGSVQ	SEQ ID NO:256	Multiple Organ
FGGSWSL	SEQ ID NO:257	Multiple Organ
LLFGGSA	SEQ ID NO:258	Multiple Organ
MRLFGGT	SEQ ID NO:259	Multiple Organ
FGGFFMY	SEQ ID NO:260	Multiple Organ
FGGFFMY	SEQ ID NO:261	Multiple Organ
EFGGQMN	SEQ ID NO:262	Multiple Organ
TFGGLIL	SEQ ID NO:263	Multiple Organ
GNSFGGW	SEQ ID NO:264	Multiple Organ
RTFGGAG	SEQ ID NO:265	Multiple Organ
WVFGGKS	SEQ ID NO:266	Multiple Organ
RGFGGLS	SEQ ID NO:267	Multiple Organ
LWPSFGG	SEQ ID NO:268	Multiple Organ
GERISGP	SEQ ID NO:269	Multiple Organ
GERLSSR	SEQ ID NO:270	Multiple Organ
TEGERAG	SEQ ID NO:271	Multiple Organ
WWLGERV	SEQ ID NO:272	Multiple Organ
WAWAGER	SEQ ID NO:273	Multiple Organ

GVISGER	SEQ ID NO:274	Multiple Organ
GPGGERG	SEQ ID NO:275	Multiple Organ
LGGGERD	SEQ ID NO:276	Multiple Organ
DIAGERV	SEQ ID NO:277	Multiple Organ
SRSKGER	SEQ ID NO:278	Multiple Organ
KRKGERV	SEQ ID NO:279	Multiple Organ
SRPGERQ	SEQ ID NO:280	Multiple Organ
CMRRGER	SEQ ID NO:281	Multiple Organ
TLRGERN	SEQ ID NO:282	Multiple Organ
FGERNRI	SEQ ID NO:283	Multiple Organ
RGERWDL	SEQ ID NO:284	Multiple Organ
GERTALL	SEQ ID NO:285	Multiple Organ
PSGTSSW	SEQ ID NO:286	Multiple Organ
SMSGTGM	SEQ ID NO:287	Multiple Organ
LFDVSGT	SEQ ID NO:288	Multiple Organ
VTGLSGT	SEQ ID NO:289	Multiple Organ
NMVISGT	SEQ ID NO:290	Multiple Organ
GVSGLTG	SEQ ID NO:291	Multiple Organ
RSGLPGK	SEQ ID NO:292	Multiple Organ
GRSGTSG	SEQ ID NO:293	Multiple Organ
IYSGTLW	SEQ ID NO:294	Multiple Organ
CSGTLFC	SEQ ID NO:295	Multiple Organ
RSGLQT	SEQ ID NO:296	Multiple Organ

LSGTWS	SEQ ID NO:297	Multiple Organ
ESGTATG	SEQ ID NO:298	Multiple Organ
FTERSGT	SEQ ID NO:299	Multiple Organ
RYLRSGT	SEQ ID NO:300	Multiple Organ
PLGSSGT	SEQ ID NO:301	Multiple Organ

The tripeptide motifs listed in Table 3 above were identified using a one-tail Fisher's test. The identification of statistically significant human targeting peptide motifs was dependent in part on the type of statistical analysis applied. Using a two-tail Fisher's test, a somewhat different set of tripeptide motifs was identified, shown in Table 6.

Table 6. Human tripeptide motifs identified using a two-tail Fisher's test

Tissues	Motifs	Target organ vs. other organs	Target organ vs. unselected library
Adipose tissue	EVS (SEQ ID NO:302)	0.0059	0.0297
	TAG (SEQ ID NO:303)	0.0022	0.0018
	VTV (SEQ ID NO:304)	0.0012	0.0073
	GEV (SEQ ID NO:305)	0.0397	0.0147
	LPG (SEQ ID NO:306)	0.0242	0.0297
	LSP (SEQ ID NO:8)	0.0040	0.0147
	PGL (SEQ ID NO:307)	0.0402	0.0297
	RGT (SEQ ID NO:308)	0.0331	0.0367
	VLL (SEQ ID NO:309)	0.0204	0.0297
Skeletal muscle	PAV (SEQ ID NO:310)	0.0317	0.0255
	AAV (SEQ ID NO:311)	0.0100	0.0255
	LVS (SEQ ID NO:10)	0.0042	0.0031
	RGP (SEQ ID NO:312)	0.0488	0.0255
	VRM (SEQ ID NO:313)	0.0015	0.0255
Prostate	LLL (SEQ ID NO:314)	0.0056	0.0169
	RAV (SEQ ID NO:315)	0.0040	0.0084
	RGE (SEQ ID NO:316)	0.0433	0.0324
Skin	ARL (SEQ ID NO:21)	0.0444	0.0080
	KGG (SEQ ID NO:317)	0.0225	0.0317
	GAR (SEQ ID NO:318)	0.0016	0.0004

	GGR (SEQ ID NO:319)	0.0001	0.0262
	GRR (SEQ ID NO:12)	0.0284	0.0322
	WGG (SEQ ID NO:320)	0.0172	0.0078
Bone marrow	GGG (SEQ ID NO:5)	0.0469	0.0070
	GGM (SEQ ID NO:321)	0.0404	0.0067
	GVD (SEQ ID NO:322)	0.0182	0.0183
	LGF (SEQ ID NO:323)	0.0062	0.0183
	SWV (SEQ ID NO:324)	0.0388	0.0183
	SWR (SEQ ID NO:325)	0.0388	0.0183

Several tripeptide motifs were identified as significant by either statistical analysis (indicated in bold in Table 6). While the two statistical analyses identified somewhat different sets of tripeptide motifs, it is considered that tripeptide sequences identified by either statistical analysis will be of use for targeting applications within the scope of the present invention.

The skilled artisan will realize that the multiple targeting peptide sequences identified in the present Example will be of use for numerous applications within the scope of the present invention, including but not limited to targeted delivery of therapeutic agents or gene therapy, *in vivo* imaging of normal or diseased organs, tissues or cell types, identification of receptors and receptor ligands in organs, tissues or cell types, and therapeutic treatment of a number of human diseases.

Example 3. Polyorgan Targeting

In standard protocols, developed with animal model systems, targeting phage are collected from a single organ, tissue or cell type, amplified and reinjected into a second animal, then recollected from the same organ, tissue or cell type. With humans this process is inefficient. In certain alternative embodiments, the efficiency of human organ, tissue or cell type targeting may be improved by polyorgan targeting, in which

phage may be collected from multiple organs, tissues or cell types of a first human, pooled and injected into a second human.

It is possible to perform multiple rounds of selection in humans if the phage recovered from each biopsy is prepared for reinjection as a pool. This protocol has been successfully demonstrated in the mouse model, using the following exemplary procedure.

A phage library (CX7C) was intravenously injected into anesthetized mice. The input was approximately 10^{10} transducing units. After 15 minutes and no perfusion (to emulate the human protocol), a variety of organs were removed, including brain, kidney, pancreas, uterus, skeletal muscle, and intestine. After grinding with a Dounce glass homogenizer, tissue samples were washed. After washing, the tissues are incubated with starved competent *E. coli* K91 and serial dilutions of bacteria were plated on LB tet/kan plates or dishes containing 40 $\mu\text{g/ml}$ of tetracycline and 100 $\mu\text{g/ml}$ kanamycin. Different dilutions for each organ sample were plated, as disclosed above. The dishes were then incubated overnight at 37°C. After the desired enrichment of the phage pool to a certain organ was obtained, about 50 phage clones from the last two rounds of selection were sequenced. Encoded peptide sequences were compared to identify enriched peptides or peptide motifs. The selectivity of selected clones was validated by individually comparing their homing to other organs and also to the homing of an insertless phage clone.

The remaining 2-3 ml of infected culture (including the homogenized tissue) were transferred to 10 ml of LB medium containing 40 $\mu\text{g/ml}$ tetracycline and 100 $\mu\text{g/ml}$ kanamycin (LB tet/kan) and shaken at 37°C for 2 hr. This approximately 12 ml culture was transferred to 1 liter of LB tet/kan and grown overnight in the 37°C shaker. The next day, phage were rescued from the bulk amplified bacterial culture according to standard protocols. These samples were pooled and reinjected for the polyorgan targeting.

In the second and third rounds of selection, 50-100 microliters out of 1 ml of the total bulk phage recovered from each organ was pooled. The resulting mixture was

reinjecting in another mouse and the same organs were recovered after 10 minutes (no perfusion). Plating of the phage from each organ revealed enrichment for each site except uterus, showing that selection was possible with the polyorgan protocol. Progressive enrichment of targeting phage in other organs as a function of the number of rounds of polyorgan targeting (not shown).

Peptide motifs of phage targeting skeletal muscle and kidney by polyorgan targeting are listed in Table 7 below.

Table 7. Consensus Peptide Motifs Identified by Polyorgan Targeting in Mice

Skeletal Muscle

VG V	SEQ ID NO:326
VG VG	SEQ ID NO:327
IG S	SEQ ID NO:328
GGA	SEQ ID NO:329
VGA	SEQ ID NO:330
WHG	SEQ ID NO:331
SGEA	SEQ ID NO:332
SNEA	SEQ ID NO:333
SLRD	SEQ ID NO:334
SVRD	SEQ ID NO:335
SARD	SEQ ID NO:336
HVV	SEQ ID NO:337
RTG	SEQ ID NO:422
RLG	SEQ ID NO:423
RWG	SEQ ID NO:338
LRWG	SEQ ID NO:339

RNS	SEQ ID NO:340
RSG	SEQ ID NO:341
GRSG	SEQ ID NO:342
RSGL	SEQ ID NO:343
YGR	SEQ ID NO:344
ASL	SEQ ID NO:345
SGA	SEQ ID NO:346
SGR	SEQ ID NO:347
DSG	SEQ ID NO:348
SSRV	SEQ ID NO:349
SSRI	SEQ ID NO:350
FGSR	SEQ ID NO:351
FCSR	SEQ ID NO:352
FQS	SEQ ID NO:353
PPV	SEQ ID NO:354
LFQ	SEQ ID NO:355
VVA	SEQ ID NO:356
AVV	SEQ ID NO:357
VAS	SEQ ID NO:358
EVSFRR	SEQ ID NO:359
EPSLFLR	SEQ ID NO:360
PGL	SEQ ID NO:361
PGI	SEQ ID NO:362
GLA	SEQ ID NO:363

VGLAV	SEQ ID NO:364
VGPAV	SEQ ID NO:365
TRG	SEQ ID NO:366
GLS	SEQ ID NO:367
DWR	SEQ ID NO:368
RGA	SEQ ID NO:369
RGG	SEQ ID NO:370
ARGG	SEQ ID NO:371
NGR	SEQ ID NO:372
ALAKG	SEQ ID NO:373
ALARG	SEQ ID NO:374
TTQ	SEQ ID NO:375
GYR	SEQ ID NO:376
GYL	SEQ ID NO:377
DRT	SEQ ID NO:378
IYS	SEQ ID NO:379
WLS	SEQ ID NO:380
IITTES	SEQ ID NO:381
IFQTES	SEQ ID NO:382
IARP	SEQ ID NO:383
IVRP	SEQ ID NO:384
HRP	SEQ ID NO:385
LRPI	SEQ ID NO:386
KNWAHLR	SEQ ID NO:387

ASLR	SEQ ID NO:388
AGLR	SEQ ID NO:389
AAV	SEQ ID NO:390
QLDRH	SEQ ID NO:391
QWDRH	SEQ ID NO:392
VEV	SEQ ID NO:393
FRYL	SEQ ID NO:394
FRSL	SEQ ID NO:395
ARI	SEQ ID NO:396
ARL	SEQ ID NO:397
ARLG	SEQ ID NO:398
ARIG	SEQ ID NO:399
RSA	SEQ ID NO:400
ADWF	SEQ ID NO:401
ADAF	SEQ ID NO:402
GWS	SEQ ID NO:403
TWM	SEQ ID NO:404

Kidney

LPS	SEQ ID NO:405
GLP	SEQ ID NO:406
LLG	SEQ ID NO:407
LDS	SEQ ID NO:408
GLR	SEQ ID NO:409
TVS	SEQ ID NO:410

GGGR	SEQ ID NO:411
SGVR	SEQ ID NO:412
VWG	SEQ ID NO:413
VGG	SEQ ID NO:414
LRVR	SEQ ID NO:415
YVR	SEQ ID NO:416
VFG	SEQ ID NO:417
PQL	SEQ ID NO:418
RAA	SEQ ID NO:419
RWA	SEQ ID NO:420
LGS	SEQ ID NO: 421

This example showed the feasibility of polyorgan targeting in a single subject to which a mixture of organ targeting phage was administered as a pool. This method is particularly relevant for vascular targeting in humans. Due to the shortage of potential subjects and the expense of processing each one, it is impractical to inject a human with a single phage population to target a single organ at a time.

* * *

All of the COMPOSITIONS, METHODS and APPARATUS disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it are apparent to those of skill in the art that variations may be applied to the COMPOSITIONS, METHODS and APPARATUS and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it are apparent that certain agents that are both chemically and physiologically related may be

substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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WHAT IS CLAIMED IS:

1. A method of identifying a targeting peptide, comprising:
 - a) obtaining a phage display library;
 - b) injecting the phage display library into the circulation of a human;
 - c) obtaining a sample of an organ, tissue or cell type from said human; and
 - d) identifying one or more targeting peptides from phage present in said organ, tissue or cell type.
2. The method of claim 1, wherein said human is brain dead or a terminal wean patient.
3. The method of claim 1, wherein said library contains approximately 10^{14} transforming units (TU).
4. The method of claim 3, wherein said library is a primary phage library.
5. The method of claim 3, wherein said library contains at least 10^8 different targeting peptide sequences.
6. The method of claim 1, further comprising recovering phage from said organ, tissue or cell type.
7. The method of claim 6, wherein phage are recovered by infecting bacteria.
8. The method of claim 6, wherein phage are recovered by amplifying phage inserts.
9. The method of claim 8, wherein the inserts encode targeting peptides.

10. The method of claim 9, further comprising ligating the amplified inserts to phage DNA to produce new phage.
11. The method of claim 1, further comprising:
 - a) performing a statistical analysis of the targeting peptide sequences; and
 - b) identifying statistically significant peptide motifs.
12. The method of claim 11, further comprising
 - a) conducting an amino acid residue sequence count;
 - b) tracking the relative frequency of each tripeptide motif, compared to its frequency in the unselected library; and
 - c) calculating the probability of observing each relative frequency.
13. A targeting peptide identified by the method of claim 1.
14. A targeting peptide motif identified by the method of claim 12.
15. A method of identifying a targeting peptide, comprising:
 - a) injecting a phage display library into the circulation of a human;
 - b) obtaining samples of multiple organs, tissues or cell types from said human;
 - c) recovering phage from said samples;
 - d) amplifying said phage;

- e) pooling the amplified phage;
 - f) injecting the pooled phage into the circulation of a second human;
 - h) obtaining samples of the same organs, tissues or cell types as were obtained from the first human; and
 - i) recovering phage from said samples.
16. An isolated peptide of 100 amino acids or less in size, comprising at least 3 contiguous amino acids of a sequence selected from any of SEQ ID NO:5 through SEQ ID NO:325.
17. The isolated peptide of claim 16, wherein said peptide is 50 amino acids or less in size.
18. The isolated peptide of claim 16, wherein said peptide is 25 amino acids or less in size.
19. The isolated peptide of claim 16, wherein said peptide is 10 amino acids or less in size.
20. The isolated peptide of claim 16, wherein said peptide is 7 amino acids or less in size.
21. The isolated peptide of claim 16, wherein said peptide is 5 amino acids or less in size.
22. The isolated peptide of claim 16, wherein said peptide is attached to a molecule.
23. The isolated peptide of claim 22, wherein said molecule is a drug, a chemotherapeutic agent, a radioisotope, a pro-apoptosis agent, an anti-

angiogenic agent, a hormone, a cytokine, a growth factor, a cytotoxic agent, a peptide, a protein, an antibiotic, an antibody, a Fab fragment of an antibody, an imaging agent, an antigen, a survival factor, an anti-apoptotic agent or a hormone antagonist.

24. The isolated peptide of claim 23, wherein said pro-apoptosis agent is selected from the group consisting of gramicidin, magainin, mellitin, defensin, cecropin, (KLAKLAK)₂ (SEQ ID NO:1), (KLAKKLA)₂ (SEQ ID NO:2), (KAAKKAA)₂ (SEQ ID NO:3) and (KLGKKLG)₃ (SEQ ID NO:4).
25. The isolated peptide of claim 23, wherein said anti-angiogenic agent is selected from the group consisting of thrombospondin, angiostatin, pigment epithelium-derived factor, angiotensin, laminin peptides, fibronectin peptides, plasminogen activator inhibitors, tissue metalloproteinase inhibitors, interferons, interleukin 12, platelet factor 4, IP-10, Gro-β, thrombospondin, 2-methoxyoestradiol, proliferin-related protein, carboxyamidotriazole, CM101, Marimastat, pentosan polysulphate, angiopoietin 2 (Regeneron), interferon-alpha, herbimycin A, PNU145156B, 16K prolactin fragment, Linomide, thalidomide, pentoxifylline, genistein, TNP-470, endostatin, paclitaxel, Docetaxel, polyamines, a proteasome inhibitor, a kinase inhibitor, a signaling inhibitor, SU5416, SU6668, accutin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 and minocycline.
26. The isolated peptide of claim 23, wherein said cytokine is selected from the group consisting of interleukin 1 (IL-1), IL-2, IL-5, IL-10, IL-11, IL-12, IL-18, interferon-γ (IF-γ), IF-α, IF-β, tumor necrosis factor-α (TNF-α), or GM-CSF (granulocyte macrophage colony stimulating factor).
27. The isolated peptide of claim 16, wherein said peptide is attached to a macromolecular complex.

28. The isolated peptide of claim 27, wherein the macromolecular complex is a virus, a bacteriophage, a bacterium, a liposome, a microparticle, a magnetic bead, a yeast cell, a mammalian cell or a cell.
29. The isolated peptide of claim 16, wherein said peptide is attached to a eukaryotic expression vector.
30. The isolated peptide of claim 16, wherein said vector is a gene therapy vector.
31. A fusion protein, comprising at least 3 contiguous amino acids selected from any of SEQ ID NO:5 through SEQ ID NO:325.
32. A composition comprising the isolated peptide of claim 16 in a pharmaceutically acceptable carrier.
33. The composition of claim 32, wherein said peptide is attached to a drug, a chemotherapeutic agent, a radioisotope, a pro-apoptosis agent, an anti-angiogenic agent, a hormone, a cytokine, a growth factor, a cytotoxic agent, a peptide, a protein, an antibiotic, an antibody, a Fab fragment of an antibody, an imaging agent, an antigen, a survival factor, an anti-apoptotic agent or a hormone antagonist.
34. The composition of claim 32, wherein said peptide is attached to a virus, a bacteriophage, a bacterium, a liposome, a microparticle, a magnetic bead, a yeast cell, a mammalian cell, a eukaryotic expression vector or a cell
35. A kit comprising:
- a) the isolated peptide of claim 16; and
 - b) a control peptide.

36. An antibody that selectively binds to an isolated peptide of a sequence selected from any of SEQ ID NO:5 through SEQ ID NO:325.
37. A gene therapy vector, wherein the vector expresses a targeting peptide sequence as part of a surface protein, the targeting peptide comprising at least three contiguous amino acids selected from any of SEQ ID NO:5 through SEQ ID NO:325.
38. An isolated nucleic acid of 300 nucleotides or less in size, said nucleic acid encoding a peptide according to claim 16.
39. A vector comprising the isolated nucleic acid of claim 38.
40. The vector of claim 39, wherein said vector is a plasmid, a cosmid, a yeast artificial chromosome (YAC), a bacterial artificial chromosome (BAC), a virus or a phage.
41. A method of treating a disease state comprising:
- a) selecting a peptide according to claim 1, wherein said peptide is targeted to cells associated with said disease state;
 - b) attaching to said peptide one or more molecules effective to treat said disease state; and
 - c) administering said peptide to a subject with said disease state.
42. The method of claim 41, wherein said disease state is selected from the group consisting of diabetes, inflammatory disease, arthritis, atherosclerosis, cancer, autoimmune disease, bacterial infection, viral infection, cardiovascular disease or degenerative disease.

43. The isolated peptide of claim 20, wherein said sequence is selected from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 or SEQ ID NO:130.

44. The isolated peptide of claim 20, wherein said sequence is selected from SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45 or SEQ ID NO:131.

45. The isolated peptide of claim 20, wherein said sequence is selected from any of SEQ ID NO:46 through SEQ ID NO:65.

46. The isolated peptide of claim 20, wherein said sequence is selected from any of SEQ ID NO:66 through SEQ ID NO:83.

47. The isolated peptide of claim 20, wherein said sequence is selected from any of SEQ ID NO:84 through SEQ ID NO:129.

48. The isolated peptide of claim 20, wherein said sequence is selected from any of SEQ ID NO:132 through SEQ ID NO:148.

49. The isolated peptide of claim 20, wherein said sequence is selected from any of SEQ ID NO:149 through SEQ ID NO:173.

50. A method of imaging comprising:

- a) selecting a peptide according to claim 1,
- b) attaching said peptide to an imaging agent;
- c) administering said peptide and agent to a human; and

- d) obtaining an image of the human.

51. A method of diagnosing a disease state comprising:

- a) selecting a peptide according to claim 1, wherein said peptide is targeted to cells associated with a disease state;
- b) administering said peptide to a human; and
- c) determining the distribution of said peptide in said human.

52. A method, comprising the steps of:

- a) injecting a phage display library comprising about 10^8 or more targeting peptides into the circulation of a human;
- b) allowing the library to remain in the human for a period of time and under conditions such that phage bind to the human; and
- c) obtaining a sample from said human which sample comprises bound phage.

53. The method of claim 52, further comprising isolating phage bound to the sample.

54. The method of claim 52, wherein the human is a human selected from the group consisting of a brain dead human and a terminal wean human patient.

55. The method of claim 52, wherein the library comprises about 10^{14} or more transforming units (TU).

56. The method of claim 55, wherein the library is a primary phage library.

57. The method of claim 52, wherein the library comprises at least 10^9 different targeting peptide sequences.

58. The method of claim 52, further comprising recovering phage from the sample.

59. The method of claim 52, wherein the phage are attached to a detectable label.

60. The method of claim 52, wherein the sample is obtained from diseased tissue of the human after the human has died.

61. A method, comprising the steps of:

- a) injecting a phage display library into the circulation of a human, wherein the library is comprised of 10^8 or more targeting peptides and the phage are attached to a detectable label and further wherein the human has a terminal disease and a beating heart;
- b) allowing the library to remain in the human for a period of time and under conditions such that phage of library bind to the human;
- c) obtaining a sample from the human after the heart of the human is no longer beating, wherein the sample comprises bound phage; and
- d) isolating phage from the sample.

62. The method of claim 52, further comprising:

- e) performing a statistical analysis of the targeting peptide sequences; and
- f) identifying statistically significant peptide motifs.

63. The method of claim 62, further comprising

- g) conducting an amino acid residue sequence count;
- h) tracking the relative frequency of each tripeptide motif, compared to its frequency in the unselected library; and
- i) calculating the probability of observing each relative frequency.

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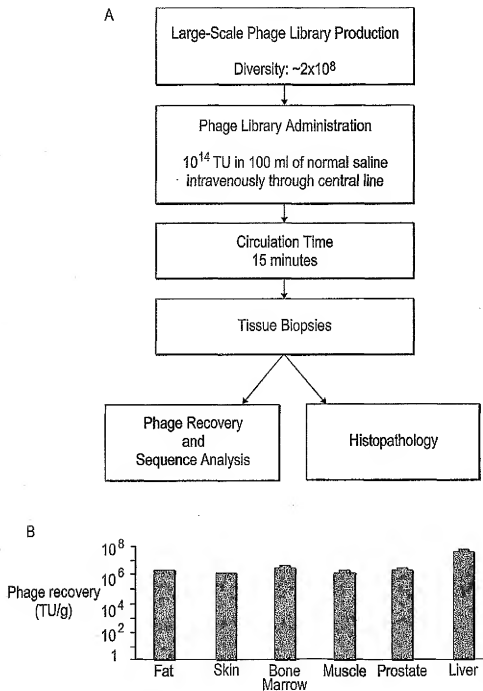


Fig. 1

2/3

Bone Marrow		Fat		Skeletal Muscle
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Prostate		Skin		
GRRAGGS TRRAGGG SRAGG-LG SYAGG-LG DVAGG-LG GAGG-LGA GAGG-WGV AGGTFCP LGEVAGG GSNDAGG YRGJAGG AGGVAGG GGLAGG-F LLAGG-VL LVVSAGG RTQAGG-V AGG-FGEQ AGG-LIDV AGGSTWT AGGDWWW AGGGLLM VAAGGGL LYGAGGS CALAGG-C IGAGGVH	GRRGSAG GRRGSAG RPGRRGS SGRRGPR GLGRRNG GGRRSQT LWDGRRH GRRSVLT FGRRNLF GAGRRYW GRRLWAT GVGRRFG LEMVGRR LSSIGRR GRRWIDV GRRREGL GRRVLGR RGLMGRR RFLLGRR PGVGRRR GVIDGRR ADGRRRLG AGRRRAQI YGRRARE PGRRLRM GGRRVTL EQGGRRRL SGRRLHP FDHSGRR GRRDVAI	GGHPRLA GGHWRVN GGHILEV GGHRAQS GDGGHRP SCVGGHS GSGVGGH VRGWGGH WRGWGGH	WGSKGTV TGS LGTV WGTVSDA ATGTVGP VV - GTVAW WVV - GTVT RVVHGTV GTVRFFS	

Fig. 2A

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Fig. 2B

PKHGVW SGLVWH GVLWAFG QARGVW GVLVSRM GTVGVV GVVLLPA GVVLLS SGVLIHD PVFSGVL FFVSGVL LLAGGV GEMGGVL GRAYGV SGVLDGR WSGGVH WSGGVH SRQGVLR RVGVLS LGVLSYR KRGVLGW FLVGLGR EGVLETS WVGWVIG VWSRGVL GVLRGVS S-FGVLRG KGSYGV GGHFGVL WMDYGV AFRVGV GVGVLRK	MEGRGAG SEGRGFM VEGRNSK VEGRYTP FN-EGRQN FEGRSRS DHVEGR WDTEGR LDWREGR RGCEGRV MTEGRV RLEGRV R-EGRRMC TOFEGR SMEGRMF PGSAEGR GEGRLA EGRFSAW EGRSDIW EGRARWL EGRRWR	QCCGFGV RGGFGVR AVGFGVI VVGFGVI GNGFGV DEFGVA WVFGV-S WFGVSL-S SMRFGVS RFGVWT-G RFGVGR-V SGLEFGV MKGVFGV AFGWSD LYAFGW KVFGWE FGVRTDL TIFGVRR	VMPREFG SRFGERV MKFGGRL RFGGALR ERFGG-DE FEGSVQ WFGSVQ LLFGGSA MRLFGGT FEGFFMY FEGFFMY EFGG-QMIN TFGGUL GNSFGGW RTFGAG WVFGGS RFGGSL LWPSFEG	GERISGP GERLSSR TEGERAG W-WLGERV WAWAGER GVISGER GPGGERG LG-GGERD DIAGERV SRSGER KR-XGERV SR-PGERQ CMR-RGER TLRGERN F-GERNRI RGERWDL GERTALL	PSGTSSW SMSGTGM LFDVSGT VTGLSGT NMVSGT GVISGTLG BSGTGPK GRSGTSG TVSGTLW CSGTLFC RSGTLQT LGSGTWS ESGTATG FTERSGT RYLRSGT PLGSSGT
--	--	--	---	--	--

Multiple Organs

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<141> 2001-09-07

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